ABSTRACT OF THESIS

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Title of Thesis The Action of Neurotropic Peptide Analogues of the ACTH Molecule.

Conditioned avoidance behaviour of rats appears to be influenced by pituitary hormones. Removal of the pituitary gland causes a marked impairment of active avoidance behaviour. Treatment of hypophysectomized rats with ACTH restores the performance towards the level displayed by intact animals (de Wied, 1962). The heptapeptide $ACTH_{4-10}$ has a similar effect but has no endocrine or peripheral metabolic activities suggesting that the behavioural influence of ACTH and $ACTH_{4-10}$ is due to an extra adrenal effect, presumably located in the central nervous system. In the intact rat, $ACTH_{4-10}$ delays the extinction of the conditioned avoidance response while $ACTH_{4-10}$-7-D-phe, where the phenylalanine residue is in the dextrorotatory form, has the opposite effect.

The object of the work described in this thesis was to investigate the mode of action of ACTH peptides using biochemical techniques in the hope that these studies might be related to the behavioural studies.

The general biochemical techniques used throughout the thesis are described in Section 1.

$ACTH_{4-10}$ was shown to increase the incorporation of ^{14}C-leucine into brain stem protein <u>in vivo</u> while having no effect on brain cortex or liver. $ACTH_{4-10}$-7-D-phe tended to decrease the incorporation into the same brain area (Section 2). These changes were visible between 1 and 4 hour after precursor injection. Subcellular fractionation of brain tissue indicated that leucine was not incorporated into the protein of one particular fraction but was distributed throughout the cell (Section 3). The mechanisms by which $ACTH_{4-10}$ could influence brain protein metabolism were investigated.

$ACTH_{4-10}$ could stimulate nucleic acid metabolism but, as it has no effect on RNA synthesis, RNA polymerase or polysome profiles (Dewar, 1972, Gispen and Schotman, 1970), this

mechanism was eliminated. $ACTH_{4-10}$ treatment had no effect on the free pools of amino acids in plasma and brain stem (Section 4) suggesting that it does not increase leucine incorporation by increasing the availability of amino acids. $ACTH_{4-10}$ did increase the incorporation of leucine into brain stem slice protein, *in vitro* while having no effect on the uptake of leucine into the slice (Section 5II). This suggests that $ACTH_{4-10}$ acts directly on protein synthesis. $ACTH_{4-10}$-7-D-phe had no effect on brain stem protein synthesis *in vitro* (Section 5II) and neither $ACTH_{4-10}$ nor $ACTH_{4-10}$-7-D-phe had any effect on retinal protein synthesis *in vitro* (Section 5 I). The significance of these results is discussed.

Brain Na^+,K^+-ATPase has been implicated in the conditioned avoidance response (Stefanovic et al., 1974) but $ACTH_{4-10}$ treatment had no effect on ATPase activity in the hypothalamus, pons or cortex, or in synaptosomes prepared from the brain stem (Section 6).

Acute injections of ACTH and MSH have been shown to increase brain electrical activity (Terda and Wolff, 1952; Sandman et al., 1971) but a single injection of $ACTH_{4-10}$ even in a dose of 2 mg/kg had no effect on rat brain electrical activity (Section 7). A series of daily injections of $ACTH_{4-10}$ reduced the increased electrical activity and spiking produced by a cortical implant of cobalt (Section 7).

The significance of these results and the possible mode of action of $ACTH_{4-10}$ are discussed in the General Discussion.

THE ACTION OF NEUROTROPIC PEPTIDE ANALOGUES OF THE ACTH MOLECULE

by

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Thesis submitted for the degree of
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Summary

Conditioned avoidance behaviour of rats appears to be influenced by pituitary hormones. Removal of the pituitary gland causes a marked impairment of active avoidance behaviour. Treatment of hypophysectomized rats with ACTH restores the performance towards the level displayed by intact animals (de Wied, 1969). The heptapeptide $ACTH_{4-10}$ has a similar effect but has no endocrine or peripheral metabolic activities suggesting that the behavioural influence of ACTH and $ACTH_{4-10}$ is due to an extra adrenal effect, presumably located in the central nervous system. In the intact rat, $ACTH_{4-10}$ delays the extinction of the conditioned avoidance response while $ACTH_{4-10}$-7-D-phe, where the phenylalanine residue is in the dextrorotatory form, has the opposite effect.

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incorporated into the protein of one particular fraction but was distributed throughout the cell (Section 3). The mechanisms by which $ACTH_{4-10}$ could influence brain protein metabolism were investigated.

$ACTH_{4-10}$ could stimulate nucleic acid metabolism but, as it has no effect on RNA synthesis, RNA polymerase or polysome profiles (Dewar, 1972, Gispen and Schotman, 1970), this mechanism was eliminated. $ACTH_{4-10}$ treatment had no effect on the free pools of amino acids in plasma and brain stem (Section 4) suggesting that it does not increase leucine incorporation by increasing the availability of amino acids. $ACTH_{4-10}$ did increase the incorporation of leucine into brain stem slice protein, <u>in vitro</u> while having no effect on the uptake of leucine into the slice (Section 5 II). This suggests that $ACTH_{4-10}$ acts directly on protein synthesis. $ACTH_{4-10}$-7-D-phe had no effect on brain stem protein synthesis <u>in vitro</u> (Section 5II) and neither $ACTH_{4-10}$ nor $ACTH_{4-10}$-7-D-phe had any effect on retinal protein synthesis <u>in vitro</u> (Section 5 I). The significance of these results is discussed.

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C O N T E N T S

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GENERAL INTRODUCTION

(1) BEHAVIOURAL STUDIES

It is well established that the pituitary adrenal system plays an essential role in the adaption of behaviour towards environmental stimuli. In the stress situation, there is a discharge of adrenocorticotrophic hormone (ACTH) from the adenohypophosis followed by an increase in the production of adrenal corticosteroids that prepare the peripheral system to overcome the stressor. In view of the psychological aberrations that are often observed in patients with adrenocortical dysfunction (Engel and Margolin, 1941) and the frequency with which adrenocortical dysfunction is observed in patients with psychiatric symptoms (Pincus, 1950), it seems that there is also some relationship between pituitary-adrenal system and behaviour. Administration of ACTH or corticosteroids frequently results in deviant behaviour (Cleghorn, 1952; Rome and Braceland, 1951). Most patients given ACTH or cortisone respond with a variety of mood and behavioural changes from feelings of well being to frank psychosis, these changes often being independent of the alleviation of any particular physiological disorder (Rome and Braceland, 1951).

Animal studies suggest that steroids modify behaviour (van Wimersma Greidanus, 1970) and that ACTH itself has a role in adaptive behaviour.

The pituitary-adrenal system appears to play an essential role in conditioned avoidance behaviour

(de Wied, 1969). In rats, removal of the whole pituitary gland or the adenohypophysis alone causes a marked impairment of active avoidance learning as measured in the pole jump or shuttle box tests (Applezweig and Baudry, 1955; Applezweig and Moeller, 1959; de Wied, 1964; de Wied, 1969; de Wied et al., 1972), while administration of adrenocorticotrophic hormone (ACTH) improves the rate of avoidance acquisition of hypophysectomized animals (Applezweig and Baudry, 1955) or adenohypophysectomized rats (de Wied, 1964) to almost normal levels.

The behavioural effect of ACTH does not seem to be mediated by the adrenal cortex as adrenalectomized rats are similar to sham-operated controls in their ability to acquire the avoidance response (Miller and Ogawa, 1962). Additional evidence has come with the use of the synthetic analogues of ACTH:- $ACTH_{1-10}$ and $ACTH_{4-10}$. Treatment of hypophysectomized rats with these peptides also restores the acquisition performance in a shuttle box to normal even though the peptides do not appear to have any corticotrophic activities (de Wied, 1969; de Wied et al., 1970). When $ACTH_{1-10}$ or $ACTH_{4-10}$ are given to hypophysectomized rats there is no change in adrenal weight, plasma corticosterone or thymus weight. Chronic peptide treatment does not influence the atrophy of the testes found in hypophysectomized rats, suggesting that it has no effect on the gonads. It does not change the body weight

loss or plasma glucose and plasma insulin levels which are decreased after hypophysectomy. This evidence suggests that $ACTH_{1-10}$ and $ACTH_{4-10}$ lack the endocrine and metabolic effects of ACTH and therefore that their behavioural effects are not mediated by corticosteroids.

The thyroid has also been implicated in acquisition and retention of the conditioned avoidance response as well as spontaneous behaviour (Bayrs and Levine, 1963). Although ACTH analogues stimulate the thyroid (Bowers et al., 1964), de Wied and Pirie (1968) have shown that the action of $ACTH_{1-10}$ in inhibiting the extinction of the conditioned avoidance response is independent of thyroid action.

It could be argued that the behavioural deficiency of the hypophysectomized rat is linked to the metabolic derangements and physical weakness that occur as a result of hypophysectomy. To some extent this is probably true. Replacement therapy consisting of cortisone, testosterone and thyroxine improves the rate of acquisition of the avoidance response and also improves the motor and/or sensory capacities of the hypophysectomized animal (de Wied, 1964). However, $ACTH_{1-10}$ and $ACTH_{4-10}$ do not improve the physical condition or metabolic status of the hypophysectomized rat but do restore the rate of acquisition of a conditioned avoidance response to almost normal levels, suggesting that they act by a more direct mechanism. In addition, $ACTH_{1-10}$ and $ACTH_{4-10}$ modify behaviour in the intact rat in that they delay the

extinction of the conditioned avoidance response (Bohus and de Wied, 1966; Greven and de Wied, 1973).

As radioactively labelled Org 2766, a small peptide which produces the same behavioural effects as $ACTH_{1-10}$, has been shown to enter the brain intact (Jansz, personal communication) we may assume that the other ACTH analogues do.

It seems possible that the peptides are acting directly on central nervous structures as in intact rats both subcutaneous and intracerebral administration of peptide retards the extinction of a conditioned avoidance response (van Wimersma, Greidanus and de Wied, 1971). Indirect evidence comes from the information that ACTH and sometimes just the N-terminal sequences are able to alter various aspects of nerve cell functioning, including neuronal firing (Sawyer et al., 1968; Krivoy, 1970; Steiner, 1970; Van Delft and Kitay, 1972), electroencephalogram synchronization (Enderoczi et al., 1970) and ACTH release (Motta et al., 1965).

The thalamic parafascicular area seems to be the possible site of action of the ACTH peptides. MSH, which contains the series of amino acids $ACTH_{4-10}$, delays the extinction of conditioned avoidance behaviour in intact rats but in rats with bilateral lesions in the thalamic parafascicular area it fails to do so (Bohus and de Wied, 1967). Intracerebral implantation studies also indicate that the parafascicular nuclei and the rostral parts of the mesencephalon are involved (van Wimersma Greidanus and de Wied, 1971).

It is possible that the behavioural effects of the peptides could be due to an effect on the general activity level of the rat. This seems unlikely as it has been shown that hypophysectomized rats are more active in exploratory behaviour than sham operated controls (Gispen et al., 1973) and that peptide treatment has no effect on ambulation, rearing, grooming and defecation in intact rats (Bohus and de Wied, 1966).

The effect of the peptides could also be due to an increase in the sensory capacities of the rat. Gispen et al. (1970) have studied sensory capacities by measuring electric shock threshold levels. In hypophysectomized rats these levels are significantly lowered. This is perhaps surprising in view of the evidence that hypophysectomized rats show defective learning of conditioned avoidance, but is in agreement with the work of Wilcock (Wilcock, 1968; Wilcock and Broadhurst, 1967) who suggests that avoidance performance is not closely correlated with the animals' response to electric shock. $ACTH_{1-10}$ does not influence the pain thresholds for electric shock even though the learning capacity is markedly enhanced, suggesting that the stimulatory effect of $ACTH_{1-10}$ cannot be explained by an effect on sensory capacities.

Both $ACTH_{4-10}$ and $ACTH_{1-10}$ are capable of increasing the rate of avoidance acquisition in hypophysectomized rats (de Wied, 1969) and decreasing the rate of avoidance extinction in intact rats (Greven and de Wied, 1967, 1973) in a way similar to a comparable dose ACTH. $ACTH_{4-7}$ is

reported to be the shortest peptide which has the essential elements required for the behavioural effects (Greven and de Wied, 1973). All the amino acid residues in the peptides referred to above are in the levorotatory form. If the phenylalanine residue in the 7 position of ACTH is changed to the dextrorotatory form different effects are observed. $ACTH_{4-10}$-7-D-phe and $ACTH_{1-10}$-7-D-phe have the opposite effects to their all-L counterparts in that they have been shown to deteriorate avoidance acquisition in hypophysectomized rats and facilitate avoidance extinction in intact rats (de Wied, 1969; Bohus and de Wied, 1966; Greven and de Wied, 1973).

In summary, it appears that ACTH, $ACTH_{1-10}$ and $ACTH_{4-10}$ change behaviour in rats, probably by a specific central action.

(2) BIOCHEMICAL STUDIES

It has been demonstrated in rats that after hypophysectomy there is a decreased synthesis of macromolecules in peripheral organs such as the liver (Korner, 1965; Tata, 1967). The content of large polysomes in the rat liver is reduced (Korner, 1964; Staehelin, 1965; Tata and Williams-Ashman, 1967) which can be explained by the decrease in RNA synthesis (Korner, 1964, 1965; Gupta and Talwar, 1968) and the increase in RNA breakdown as a result of high RNAse activity (Brewer et al., 1969). There is also evidence that hypophysectomy would lead to a defect at the ribosomal level disrupting regular binding capacity

to mRNA or to aminocyl-tRNA (Staehelin, 1965; Garren et al., 1967; Korner, 1968, 1969; Liew and Korner, 1969; Barden and Korner, 1968), although there is some dispute about this (Foster and Sells, 1969).

These changes in RNA metabolism in turn modify protein synthesis. After hypophysectomy there is a reduction of incorporation of amino acids into peptide chains resulting in a decrease of total protein, (Korner, 1968, 1969; Tata and Williams-Ashman, 1967; Cheek and Graystone, 1969; Clemens and Korner, 1970).

It seems that treatment with growth hormone is sufficient to restore the reduced synthesis of macromolecules found in the hypophysectomized rat to normal levels (Tata, 1968; O'Malley, 1968; Brewer et al., 1969; Clemens and Korner, 1970; McDonald and Korner, 1971).

In the brain it has been shown that amino acid incorporation is decreased in cell-free systems from brain tissue of hypophysectomized animals (Dunn and Korner, 1966), and that the RNA/DNA ratio in the brain stem is reduced after hypophysectomy (De Vellis and Inglish, 1968). Gispen further localized this effect by measuring the total cell RNA in 16 brain areas (Gispen et al., 1972). The RNA was reduced mainly in the brain stem structures, i.e. thalamus, hypothalamus, mesencephalon and medulla oblongata, but also in the rostral cortex. After hypophysectomy there is a significant decrease in the content of large polysomes in the rat brain stem, and a reduction of incorporation of uridine into brain total, nuclear and

cytoplasmic RNA and into polysomes (Gispen et al., 1970).

It has been suggested that, in general, brain RNA and protein metabolism are involved in the consolidation of newly acquired information (Glassman, 1969; Hyden and Lange, 1970; Shashova, 1970). Interest has therefore focused on the effect on brain macromolecule metabolism of procedures that effect behaviour. It has been shown that there is an increase in the number of large polysomes in the rat brain as a result of animal training (Dellweg, 1968) and that training increases the incorporation of uridine into brain RNA in normal and hypophysectomized rats (Glassman, 1970). As stated above, hypophysectomy impairs avoidance learning in a shuttlebox but ACTH or its analogues can restore performance to almost normal levels. It has been found that there is an increase in the fraction of large polysomes in the brain in association with an increase in conditioned avoidance learning in hypophysectomized rats treated with ACTH peptides as compared with untreated hypophysectomized rats who were unable to learn the task (Gispen and Schotman, 1970; Gispen et al., 1971). Peptide treatment without associated training procedure has no effect on the polysome pattern, suggesting that peptide treatment alone has no influence on the metabolism of large polysomes and that the above changes are due to training. These effects of training appear to be specific to the brain as no changes in polysome pattern have been observed in the liver.

It has been shown that treatment of hypophysectomized rats with $ACTH_{1-10}$ has no effect on the incorporation of uridine into rapidly labelled RNA of the brain stem (Schotman et al., 1972) and that treatment of intact rats with $ACTH_{4-10}$ has no effect on orotic acid incorporation into whole brain RNA or on RNA polymerase (Reading and Dewar, 1971).

Combining the above data, it seems that $ACTH_{1-10}$ treatment effects neither the rate of RNA synthesis nor the aggregation of polyribosomes in the brain stem.

Hypophysectomy causes a decrease in the incorporation of labelled phenylalanine into brain protein (Takahshi et al., 1970) and a decrease in the incorporation of leucine into rapidly labelled protein in the rat brain stem (Gispen and Schotman, 1973). Decreases have also been observed in both phenylalanine incorporation and in poly-U-stimulated amino acid incorporation in cell-free systems prepared from brain tissue of hypophysectomized rats (Dunn and Korner, 1966). It should be noted that Sjostrand found that hypophysectomy increased the incorporation of ^{3}H leucine into protein from all subcellular fractions of the ventral horn of the spinal cord (Sjostrand, 1967) and other regions of the CNS (unpublished data cited in Sjostrand, 1967) and Frankel and VanderLaan found no change in incorporation of ^{14}C-leucine into cell free systems from the brain of hypophysectomized rats (Frankel and VanderLaan, 1972). Gispen suggests that 'the critical points to be considered are the age at which the surgery

has been performed, the brain region studied, the time between surgery and biochemical analysis and, very importantly, an appropriated test of the effectiveness of the surgery' (Gispen and Schotman, 1973). Neither Sjostrand nor Frankel describe tests for the effectiveness of the surgery.

In hypophysectomized rats, $ACTH_{1-10}$ increases the incorporation of leucine into rapidly turning over protein whereas $ACTH_{1-10}$-7-D-phe decreases it and $ACTH_{11-24}$ has no effect (Schotman et al., 1972). These results may be compared with the behavioural studies described above where treatment of hypophysectomized rats with $ACTH_{1-10}$ restores performance in a shuttlebox to normal while $ACTH_{1-10}$-7-D-phe deteriorates the acquisition of a conditioned avoidance response, and $ACTH_{11-24}$ has no effect.

In intact rats, $ACTH_{4-10}$ increases the incorporation of ^{14}C-glycine and ^{14}C leucine into brain protein, the effect being most pronounced in the 48 hr. following precursor injection. $ACTH_{4-10}$-7-D-phe has no effect (Reading and Dewar, 1971).

Jakoubek has studied the effect of ACTH on brain RNA and protein metabolism in intact mice (Jakoubek et al., 1971a, 1971b, 1970). After an extremely high dose of ACTH, there was an inhibition of uridine incorporation into RNA or spinal motor neurons and glial cell nuclei while leucine incorporation was enhanced. Jakoubek has also reported that ACTH treatment impairs leucine incorporation into proteins from whole brain slices. It is rather

difficult to interpret these results. It seems probable that the results are due to extra adrenal effects of ACTH and are caused by corticosteroids which also interact with brain metabolism.

It therefore seems that the ACTH analogues $ACTH_{4-10}$ and $ACTH_{1-10}$ change brain protein metabolism but have no effect on brain RNA metabolism.

The aim of the work described in this thesis was to investigate the mode of action of ACTH peptides, in the hope that the biochemical studies might be related to studies on the behavioural actions of ACTH and ACTH peptides. This in turn might shed some light on the biochemical basis of the behavioural patterns. In addition a clinical use for the ACTH peptides was sought.

SECTION 1

GENERAL BIOCHEMICAL METHODOLOGY

The methods described below are those used throughout the thesis Specific methods for each experiment may be found in the individual sections or in the appendix as appropriate.

(i) Choice of animals

Male, albino Wistar rats weighing 140-160 gm were used in all experiments except those described in Section 6 where male Piebald Virol Glaxo (PVG) rats weighing 200-250 gm were used.

Albino Wistar rats were, in the first instance, obtained from Charles River (UK) Ltd. but in June, 1972 Charles River were unable to provide rats in the required weight range and thereafter rats were obtained from Bush Farm, the Edinburgh University Breeding Station. PVG rats were from stock bred in the Pharmacology Department animal house.

Rats were housed in the Pharmacology Department animal house for at least 2 days prior to the start of each experiment to allow them to recover from the stress of transportation.

Rats were kept either 4 or 5 per cage, control and experimental animals being treated alike throughout. All were allowed the standard rat diet (Oxoid Modified 45B) and tap water ad libitum, and were subject to a regular cycle of 12 hr light and 12 hr dark.

Control and drug-treated rats were kept in separate cages but care was taken to keep both groups in the same conditions. Rats were killed in a set order - one control and then one drug treated rat until all the rats had been killed. This order was chosen because McArthur et al. (1971) reported that, in mice, the first animals to be killed showed a higher incorporation of ^{14}C-histidine into liver protein than did the last animals. It is quite probable that this phenomenan also applies to tracer studies in rat brain.

(ii) Injection techniques

^{14}C-leucine was administered either by intraperitoneal injection. ACTH peptides were routinely given by intraperitoneal (IP) injection.

(a) Intraperitoneal injection

^{14}C-leucine was dissolved in physiological saline to a dilution giving the appropriate amount of radioactivity in 0.2 ml. 0.2 ml of the solution was then administered intraperitoneally using a 25 guage needle and a 1 ml disposable syringe (Gillette Scimitar).

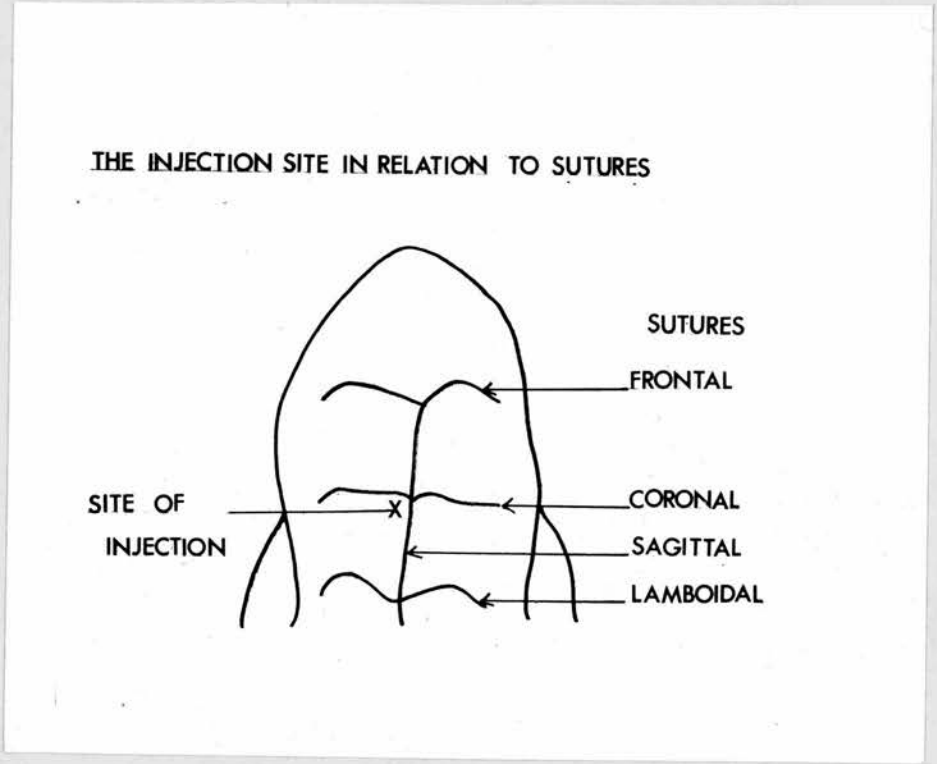
The peptides $ACTH_{4-10}$ and $ACTH_{4-10}$-D-phe were also administered by intraperitoneal injection. Usually a series of 14 daily injections of 0.2 ml of a 100 µg/ml solution was given. This is the treatment program recommended by N.V. Organon (Personal communication). Experiments were carried out to

confirm this. Ten days was found to be the minimum time to produce a change in brain protein metabolism. Fourteen days treatment was chosen to allow for any variation in response produced by different groups of rats.

(b) Intraventricular injection

In certain experiments ^{14}C-leucine was administered by intraventricular injection. The method used was based on that of Noble (Noble et al., 1967). Rats were anaesthetized by being placed in a plastic box and being subjected to 1% halothane in oxygen for approximately one minute. The rat was removed from the box and placed face downwards on a rat board. Throughout the operation anaesthetic was administered through a nose mask. A mid-line sagittal incision was made from the eyes to the ears thus exposing the skull. A hole was made in the skull 1.5 to 2 mm lateral to the intersection of the coronal and sagittal sutures (fig.1). This hole was made with a sharpened ice pick and was large enough to accommodate a 27 guage needle and just deep enough to penetrate the skull. 20 µl of the solution of the labelled precursor was injected into the lateral ventricle using a 50 µl Hamilton microsyringe fitted with a 27 guage needle. The needle had been cut leaving a length of 3.5 mm, thus limiting its depth of penetration. During the

FIG. 1


THE INJECTION SITE IN RELATION TO SUTURES
SUTURES
FRONTAL
CORONAL
SAGITTAL
LAMBOIDAL
SITE OF
INJECTION
X

injection the syringe was held in a vertical position and after injection the syringe was left in position for 10 seconds. After withdrawal, the hole in the skull was immediately sealed with bonewax to prevent leakage of the injected material and the incision was closed using Michel clips.

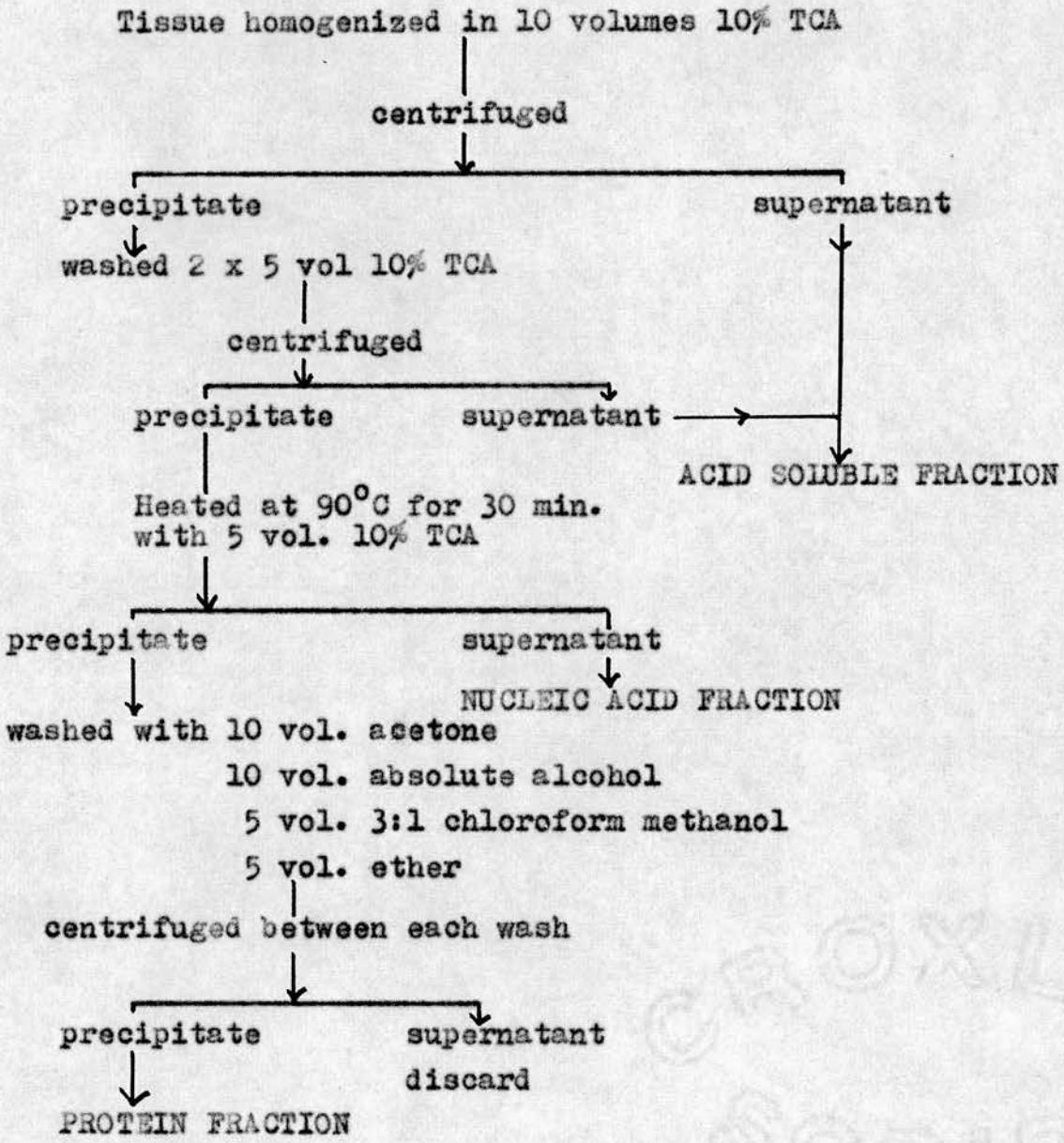
The whole procedure took from 4 to 5 minutes, the rats recovering almost instantly. Rats injected in this manner normally survived for over 2 weeks with no noticeable harmful effects.

(iii) Extraction of protein and nucleic acids

The method used was based on that of Guroff et al. (1968) as modified by Dewar (1971). A diagramatic representation of the method is given in fig.2. It depends on the differing solubilities of nucleic acids and protein in acid.

The tissue was homogenized in 10 volumes of 10% trichloroacetic acid (TCA) using a Tri-R rotary homogenizer. The clearance of the homogenizer being between 5 and 8 thou. The suspension was centrifuged at 1000 g for 10 min in a Mistral 2L centrifuge when the volume of the suspension was greater than 15 ml or for 2 min in an Eppendorf centrifuge when the volumes were less than 1.5 ml. The precipitate was washed twice with 5 vol. 10% TCA, all the supernatants being collected. The supernatants are known as the acid soluble fraction (ASF) and give a rough indication of the size of the pool of

Fig. 2 : To show the method of extraction of protein from brain tissue



free amino acids. The precipitate was then heated at 90°C in 10% TCA for 30 min. Under these conditions RNA and DNA are soluble whereas protein is not. The fractions were separated by centrifugation. As RNA does not interfere in the estimation of DNA by the methods described in the appendix, DNA could be estimated in the supernatant. The precipitate was then washed 4 times to remove lipids - once each with 10 volumes acetone, 10 vol. absolute alcohol, 5 vol chloroform: methanol (3:1) and 5 vol diethyl ether. Acetone was chosen for the first wash as it is completely miscible with water and will remove the last traces of TCAand sodium-dried diethyl ether was chosen for the last wash to dry the protein sample. The protein sample was then dry and in a suitable state for dissolving in 98% formic acid as described below (Section 1 (iv)).

(iv) Solubilization of protein

A method of solubilizing the protein was required which would allow the amount of protein to be estimated by standard biochemical techniques and would also permit the radioactivity in the fraction to be measured by liquid scintillation counting.

Both the biuret and the Lowry methods of protein estimation are sensitive to changes in pH, amines and other contaminants. Various ions can cause quenching in liquid scintillation counting and under certain conditions the protein may precipitate out in the scintillant. As the levels of radioactivity are, in most cases, rather low,

large dilutions of the protein solutions were impracticable.

Commercial solubilizers (e.g. Soluene and NCS) and hyamine were eliminated as they are amine based and interfere with the Lowry protein estimation. Many workers solubilize proteins by heating in strong alkali, which may then be neutralized and counted by scintillation counting.

A quicker method seemed to be the use of 98% formic acid as a solubilizer. Experiments were carried out to test its efficiency for scintillation counting and any possible interference on the Lowry protein estimation.

To investigate the effect of formic acid on Lowry protein estimations

Lowry protein estimations were carried out on a standard solution of protein to which varying amounts of 98% formic acid had been added.

Method

50 ml of a 2500 µg/ml solution of bovine serum albumin was prepared by dissolving 0.125 gm albumin in 50 ml distilled water. 10 ml aliquots of the standard protein solution were mixed with varying amounts of 98% formic acid (table 1). These solutions were diluted with formic acid/water dilutant (table 1), to give final concentrations of protein in the assay of 5, 10, 15 and 20 µg.

A standard Lowry protein estimation was carried out on triplicate samples of each dilution of protein by the method described in the Appendix.

The pH of the dilutant and of the protein solution plus

solution C of the Lowry estimations were measured using a pH meter (Pye-Unicam).

TABLE 1 : Volumes of formic acid added to standard protein solution

Sample Number	Volume 98% formic acid added to 10 ml protein solution (ml)	Dilutant - Volume 98% formic acid in 100 ml water (ml)
1	0	0
2	0.05	0.5
3	0.1	1.0
4	0.2	2.0
5	0.4	4.0

Results

The pH of distilled deionized water was 4.09. This was reduced to 1.7 by the addition of 0.4 ml formic acid to 100 ml water (3.92%). The pH of the protein solution plus solution C was reduced rather less (table 2).

TABLE 2 : To show the pH of various formic acid solutions and the pH after neutralization of these solutions with sodium carbonate

Sample Number	% Formic Acid in Dilutant	pH of dilutant	pH of protein solution + solution C
1	0	4.09	11.82
2	0.49	2.35	11.82
3	0.98	2.17	11.79
4	1.96	2.08	11.73
5	3.92	1.70	11.09

0.49, 0.98 and 1.96% formic acid had no significant effect on the standard curve for the Lowry protein estimation but 3.92% reduced the sensitivity of the system (fig. 3).

Discussion

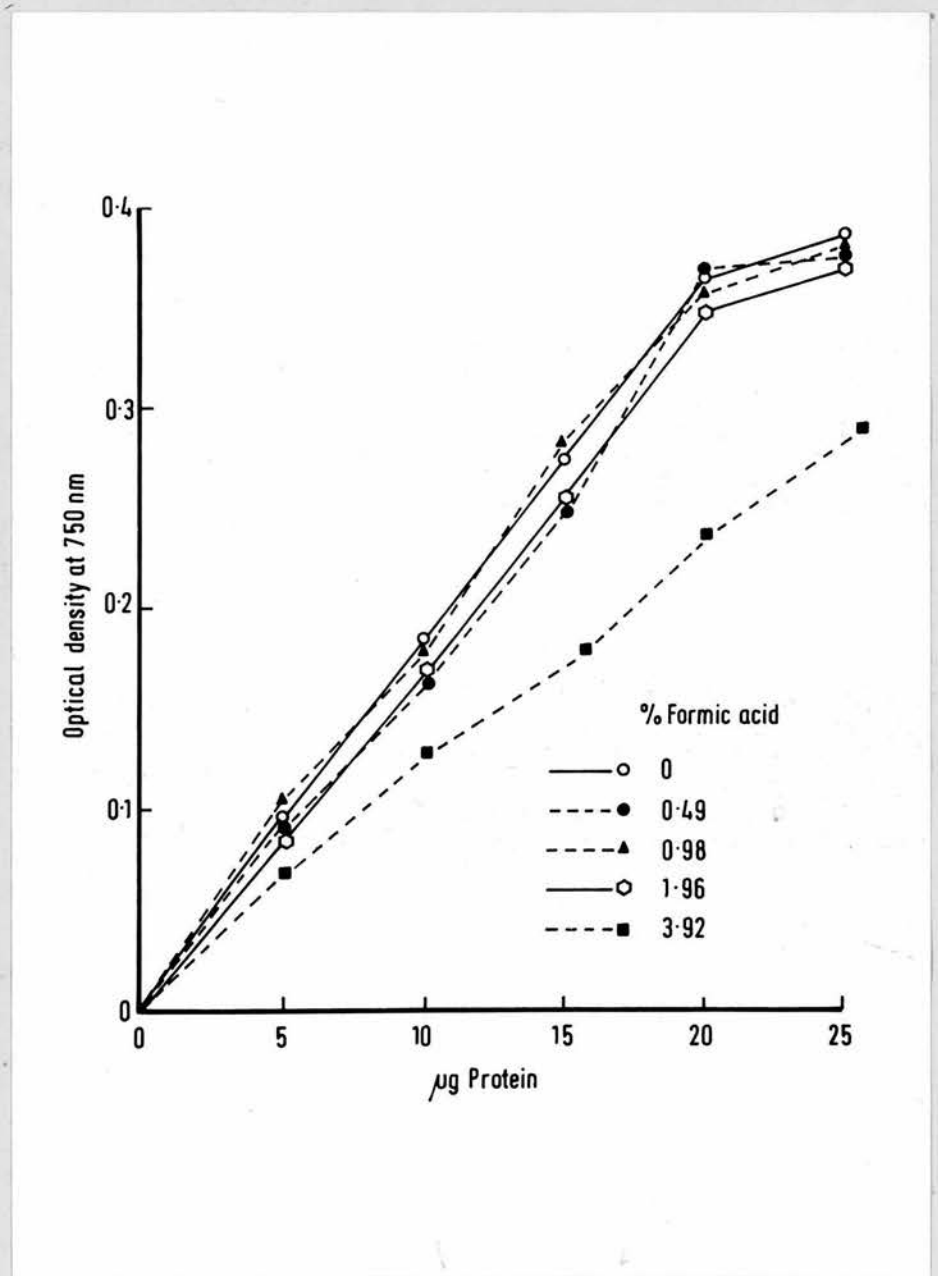
0.49, 0.98 and 1.96% formic acid had little effect on the pH of the protein solution diluted with Na_2CO_3 in NaOH (Solution C) and therefore no effect on the protein estimation while 3.92% formic acid reduced the pH and reduced the sensitivity of the assay.

As the protein/formic acid solutions in my experiments were diluted to the range 0.49 to 1.96% formic acid this was a suitable method for solubilizing protein. As an additional precaution formic acid, in the appropriate concentration was added to all standards in the experiments described below.

(v) Choice of labelled amino acid

L- 1-^{14}C leucine (Radiochemical Centre, Amersham) was used throughout. Some amino acids have pharmacological functions or are involved in the respiratory and energy producing process in the brain, but leucine is predominantly used as a constituent of protein. It is therefore suitable for use in tracer studies. The isotope carbon 14 was chosen rather than tritium as the former is not involved in exchange reactions.

Fig. 3 : To show the effect of formic acid on the Lowry protein estimation



(vi) Scintillation counting

As ^{14}C, a low energy β emitter, was used throughout, liquid scintillation counting was chosen to estimate radioactivity.

(a) Choice of scintillants

Toluene is regarded as the best solvent for the phosphors used in scintillants as it is a good transmitter of energy. Unfortunately it is unsuitable for aqueous samples as it is unable to take up more than 0.5 ml of water in 15 ml scintillant even after the addition of ethanol to the scintillant mixture. A dioxan based scintillant was used even though it is not such a good transmitter of energy as toluene. As dioxan freezes at 12°C, naphthalene was added to the mixture to depress the freezing point. The composition of the scintillant used, known as "dioxan scintillant", was as follows:

10 gm Napthalene
0.7 gm PPO
30 mg POPOP
38.5 ml xylene
38.5 ml dioxan
23.0 ml absolute alcohol

This scintillant takes up over 1 ml of water in 10 ml scintillant giving a one phase mixture. If the proportion of ethanol is increased, the scintillant is able to take up more water but this procedure also increases the degree of quenching. This scintillant

was used for all aqueous samples, i.e. the acid soluble fraction in all experiments and samples of medium obtained in the *in vitro* experiments described in Section 5.

Counting protein samples was rather more difficult. As described before, protein may be dissolved by incubating at 37°C overnight with 40% NaOH or hyamine hydroxide, but these solutions were difficult to count because of chemiluminescence. Chemiluminescence decreases with time but the protein is precipitated with time decreasing the counts even further. Formic acid was chosen as a protein solubilizer as it permitted the estimation of protein by the Lowry method and of radioactivity by liquid scintillation counting.

A high counting efficiency was obtained when the commercial scintillant 'Aquasol' was used. Unfortunately, dioxan scintillant could not be used as the counting efficiency was reduced. When 1 ml 98% formic acid is added to 15 ml dioxan scintillant the background is increased by 600%. If the formic acid is diluted 10 times, the background returns to normal but precipitation of the protein is still a problem. Aquasol (N.E.N.), a xylene based scintillant was found to be a suitable scintillant. It is able to take up a formic acid solubilized protein sample of over 1 ml with no precipitation and no elevation of background. Counting efficiency was routinely 84%.

(b) Efficiency of counting

Radioactivity was measured using a Nuclear Chicago scintillation counter. The counts were corrected for quenching using the external standard channels ratio method (Rogers and Moran, 1966). Using this method the sample is only counted once. The external standard is of very high activity requiring a short counting time. This method is therefore suitable for counting samples of low activity because a shorter total counting time is required than for other methods, although it is rather less accurate for counting highly quenched samples than an internal standard technique.

SECTION 2

THE EFFECT OF ACTH PEPTIDES ON BRAIN PROTEIN METABOLISM IN VIVO

(i) To show the effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into rapidly turning over brain protein in vivo

Introduction

In the intact rat, it has been reported that $ACTH_{4-10}$ increases the incorporation of ^{14}C-leucine and ^{14}C-glycine into brain protein while having no effect on the incorporation of ^{14}C-glycine or ^{14}C-orotic acid into cerebral RNA (Reading and Dewar, 1971) or on pancreatic ribonuclease (Reading, 1972). The effect on protein metabolism was reported to be most pronounced in the 48 hr following precursor injection, but no further experimental details or results have been reported.

In view of the effects of $ACTH_{1-10}$ on protein with a short half life in the brain stem of hypophysectomized rats (Schotman et al., 1972), it was of interest to try to localize the effect of $ACTH_{4-10}$ in intact rats to one gross brain area and to discover at what time after precursor injection the effect was maximal.

As Schotman et al. (1972) found that $ACTH_{1-10}$ stimulated the incorporation of leucine into protein of hypophysectomized rats in the 5 min after precursor injection, this time was chosen to discover if ACTH peptides had a similar effect in intact rats.

Method

Ten male albino Wistar rats were randomly assigned to one of two groups:

$ACTH_{4-10}$ Group received daily intraperitoneal injections of a neutral solution of 20 µg $ACTH_{4-10}$ in 0.2 ml physiological saline.

Control Group received daily intraperitoneal injections of 0.2 ml physiological saline.

These injections were continued throughout the experiment. On the 14th day of the experiment all rats received an intraperitoneal injection of 5µCi ^{14}C-leucine in 0.2 ml saline and were killed by decapitation 5 min later. The brain was rapidly removed and dissected into 3 gross brain areas: cerebellum, cortex and brain stem, as defined by Gispen (Gispen et al., 1972). A sample of liver was also taken. Lipid and carbohydrate-free protein was extracted from these samples and estimated as described in the methods (Section 1 (iii)).

Results

Table 1 shows that $ACTH_{4-10}$ has no effect on the incorporation of ^{14}C-leucine into brain or liver protein 5 min after injection of precursor. The results are expressed as specific activity which may be defined as the d.p.m. per mg extracted acid insoluble protein.

TABLE 1 : To show the effect of $ACTH_{4-10}$ treatment on the incorporation of ^{14}C-L-leucine into brain and liver protein synthesized 5 min after precursor injection

	ACID INSOLUBLE PROTEIN specific activity d.p.m./mg protein	ACID SOLUBLE FRACTION d.p.m./gm wet weight
CEREBELLUM		
$ACTH_{4-10}$	29.01 ± 8.79 (5)	4.06 ± 1.10 (5)
Control	21.15 ± 6.73 (5)	3.30 ± 0.46 (5)
CORTEX		
$ACTH_{4-10}$	16.5 ± 3.46 (5)	3.24 ± 0.34 (5)
Control	18.19 ± 5.13 (5)	3.39 ± 0.51 (5)
BRAIN STEM		
$ACTH_{4-10}$	19.38 ± 3.27 (5)	3.14 ± 0.29 (5)
Control	19.84 ± 5.82 (5)	3.90 ± 0.49 (5)
LIVER		
$ACTH_{4-10}$	305.7 ± 101.1 (5)	45.0 ± 7.74 (5)
Control	298.5 ± 75.1 (5)	56.9 ± 11.79 (5)

Results expressed as mean specific activity ± standard deviation. The number of animals is given in parenthesis.

The incorporation of C^{14} leucine into brain protein will be limited by quantity of labelled leucine which has entered the brain. This, in turn, will be influenced by the accuracy of the precursor injection and the uptake of leucine from the plasma by the brain and liver. The

radioactivity in the tricholoroactic acid soluble fraction gives an indication of the free amino acids and is included in table 1.

Table 2 shows the effect of $ACTH_{4-10}$ on the relative specific activity. This is defined as d.p.m. in the acid insoluble fraction/mg protein extracted/1000 ASF d.p.m. and therefore corrects for any differences due to injection error.

TABLE 2 : To show the effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into brain and liver protein. Results expressed as relative specific activity.

GROUP	
	CEREBELLUM
$ACTH_{4-10}$	22.7 ± 6.9 (5)
Control	25.1 ± 4.5 (5)
	CORTEX
$ACTH_{4-10}$	10.88 ± 2.6 (5)
Control	9.85 ± 3.3 (5)
	BRAIN STEM
$ACTH_{4-10}$	11.45 ± 3.51 (5)
Control	12.31 ± 2.07 (5)
	LIVER
$ACTH_{4-10}$	20.34 ± 12.3 (5)
Control	26.29 ± 10.04 (5)

Results expressed as mean relative specific activity ± standard deviation.

Number of animals in parenthesis.

From table 2 if may be seen that there is no significant difference between the relative specific activity of $ACTH_{4-10}$ treated animals and the controls.

Discussion

Treatment of intact rats with $ACTH_{4-10}$ has no effect on the incorporation of ^{14}C-leucine into brain stem protein with a short half life.

These results may be compared with those of Schotman (Schotman et al., 1972) who found that $ACTH_{1-10}$ stimulated the synthesis of proteins with a short half life in hypophysectomized rats.

It seems probable that in intact rats ACTH analogues are unable to upset the homeostatic controls of protein synthesis whereas in hypophysectomized rats they are able to restore the deficient protein synthesis towards normal levels. It is also possible that the differences between these and Schotman's results are due to the different modes of administration of the peptide. Schotman gives a zinc-peptide complex whereas here the peptide was administered intraperitoneally as a neutral solution.

(ii) To compare the effects of administration of $ACTH_{4-10}$ solution with $ACTH_{4-10}$-zinc complex on the incorporation of ^{14}C-leucine into brain proteins with a short half life

In zinc deficient dwarfs, exogenous doses of ACTH elicit little adrenal response but after oral zinc therapy a marked response can be measured (Standsted et al., 1966) suggesting that zinc is involved in the action of ACTH.

In rats subjected to the stress of hypotensive episodes and allowed to recover, fluctuating plasma ACTH levels are seen which are closely followed by fluctuating plasma zinc levels (Flynn et al., 1971). In vitro ACTH is unable to elicit a response in isolated adrenal slices when the zinc levels have been reduced with a zinc chelator (Flynn et al., 1972) but the response is restored by the addition of zinc. It has been postulated that zinc binds to the glutamic acid residues in ACTH and is in some way necessary for the action of ACTH on the adrenal cortex (Flynn et al., 1972). It is also possible that zinc is necessary for the action of ACTH and ACTH analogues on the central nervous system.

All commercially available ACTH contains zinc to a concentration of approximately 4 ppm/mg unit, but the synthetic peptides from Organon are reported not to contain any zinc (Organon personal communication). De Wied (de Wied, 1966) reported that 15 times as much ACTH was necessary to elicit a behavioural response when administered in partially hydrolyzed gelatine as when administered as a zinc-phosphate-ACTH complex. He stated that this was because the long acting gelatine preparation was known to possess less potent corticotrophic activities than the zinc-phosphate complex. It is conceivable that this is due to the presence of zinc in the latter preparation.

The chronic presence of ACTH and ACTH peptides is necessary to elicit a behavioural response (de Wied, 1966). The zinc-phosphate-ACTH complex is a long acting preparation

releasing ACTH continously. If the half life of the peptides in the body is short, daily intraperitoneal injections of peptide solution may not be sufficient to maintain levels of peptide high enough to produce a response.

Consequently an experiment was designed to compare the effects of $ACTH_{4-10}$ given as a neutral solution by intraperitoneal injection with $ACTH_{4-10}$ given as a zinc phosphate complex by subcutaneous injection.

Rats were killed 15 min after injection of ^{14}C-leucine rather than 5 min as in the previous experiment. This was to allow a greater degree of incorporation of leucine into protein and thereby increase the accuracy of the method. Schotman found that $ACTH_{1-10}$ stimulates protein synthesis in hypophysectomized rats both 5 and 15 min incorporation times. (Schotman et al., 1972).

Method

20 male, albino Wistar rats weighing 140-160 gm were randomly assigned to one of four groups.

$ACTH_{4-10}$ Group received daily intraperitoneal injections of a neutral solution of 20 µg $ACTH_{4-10}$ in 0.2 ml physiological saline.

CONTROL Group received daily intraperitoneal injections of 0.2 ml physiological saline.

$Zn/ACTH_{4-10}$ Group received a subcutaneous injection of 0.2 ml zinc-phosphate $ACTH_{4-10}$ complex every other day.

Zn/CONTROL Group received a subcutaneous injection of 0.2 ml of a zinc-phosphate plaecebo every other day.

The zinc complexes were prepared by the method of de Wied (1966).

On the 14th day of the experiment all rats received an intraperitoneal injection of 5 μCi ^{14}C-L-leucine in 0.2 ml saline and were killed by decapitation 15 min later. The brain was quickly removed and dissected into 3 gross brain areas, cerebellum, cortex and brain stem. A sample of liver was also taken. Lipid and carbohydrate-free protein was extracted and estimated as described before (Section 1 (iii) and Appendix).

Results

It may be seen from table 3 that neither $ACTH_{4-10}$ given as a neutral solution nor as a zinc complex has any effect on the synthesis proteins labelled after 15 min in brain or liver.

The results are expressed as specific activity, d.p.m. in the acid in soluble fraction/mg protein extracted and as relative specific activity which adjusts for any errors due to the inaccuracy of the injection. In none of the groups is there any significant difference between $ACTH_{4-10}$ treated and control animals and there is no significant difference between rats treated with $ACTH_{4-10}$ in solution or as a zinc complex.

Discussion

$ACTH_{4-10}$ has no effect on the synthesis of proteins with a very short half-life in the presence or absence of zinc in the intact rat.

TABLE 3 : To show the effect of $ACTH_{4-10}$ administered to rats by 2 different methods on the incorporation of ^{14}C leucine into protein.

	Specific Activity dpm/mg protein	Specific Activity in acid soluble fraction dpm/qm wet weight	Relative specific activity dpm/mg protein/ ASF [illegible].m.
CEREBELLUM			
$ACTH_{4-10}$	68.7±22.3(5)	[illegible]	
Control	51.0±27.9(5)	645.5±281.4(5)	28.7±19.6(5)
$Zn/ACTH_{4-10}$	58.9±19.3(5)	520.4±223.3(5)	45.3±21.7(5)
Zn/Control	51.8±17.6(5)	576.2±191.4(5)	31.4±5.8 (5)
CORTEX			
$ACTH_{4-10}$	76.2±27.9(5)	726.9±304.4(5)	13.5± 3.6(5)
Control	56.6±27.4(5)	812.8±471.0(5)	9.3± 5.3(5)
$Zn/ACTH_{4-10}$	66.0±26.5(5)	701.1±194.7(5)	11.7± 3.4(5)
Zn/Control	60.7±17.3(5)	568.4±186.1(5)	11.6± 1.4(5)
BRAIN STEM			
$ACTH_{4-10}$	54.5±18.3(5)	915.4±382.0(5)	16.7± 6.8(5)
Control	55.4± 7.8(5)	758.3±329.1(5)	18.2±11.9(5)
$Zn/ACTH_{4-10}$	66.2±27.8(5)	772.1±353.2(5)	18.2± 8.1(5)
Zn/Control	57.2±17.3(5)	545.9±148.3(5)	18.3± 4.9(5)
LIVER			
$ACTH_{4-10}$	706.3±257.1(5)	2352 ±778 (5)	72.7±45.4(5)
Control	541.6±257.1(5)	2131.3±749 (5)	73.7±15.9(5)
$Zn/ACTH_{4-10}$	585.6±235.4(5)	2195.1±767 (5)	72.5±26.7(5)
Zn/Control	593.4±165.2(5)	2559.2±1233(5)	93.9±51.4(5)

Results given as mean ± std. deviation (number of animals)

(iii) <u>To show the effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into the brain protein synthesized in the 48 hr following precursor injection</u>

In view of the report by Reading and Dewar (1971) that $ACTH_{4-10}$ stimulates the incorporation of ^{14}C leucine and ^{14}C-glycine into the brain protein of intact rats within the 48 hr following precursor injection, it seemed important to investigate the effect of $ACTH_{4-10}$ on proteins with a longer half-life.

C^{14} leucine was administered by intraventricular injection to minimize errors due to differences in absorption of leucine by the brain from the plasma.

<u>Method</u>

40 male, albino Wistar rats, weighing 140-160 gm were randomly assigned to one of two groups.

$ACTH_{4-10}$ Group received daily intraperitoneal injections of 0.2 ml of a neutral solution of $ACTH_{4-10}$ in physiological saline.

CONTROL Group received daily intraperitoneal injections of 0.2 ml of physiological saline.

On the 14th day of the experiment all rats received an intraventricular injection of 1.25 µCi ^{14}C-L-leucine in 20 µl physiological saline (see Section 1 (ii(b)). Ten rats, 5 from each group were killed 1 hr after the injection of precursor. Further groups were killed, 4, 24 and 48 hr after the leucine injection. The brain was divided into 4 areas; hind brain, brain stem and 2 half cortices. Carbohydrate and lipid free protein was

extracted and estimated as described before (Section 1(iii) and the Appendix).

Results

Tables 4 and 5 show that $ACTH_{4-10}$ treatment causes a significant increase in the incorporation of labelled leucine into protein after 1 hr. This is seen when the results are expressed as specific activity (table 4) or as relative specific activity (table 5). The amount of label in the brain stem is also increased 24 hr after precursor injection.

Discussion

$ACTH_{4-10}$ treatment stimulates the synthesis of protein in the brain stem and hind brain 1 hr after injection of precursor, and increases the level of label in the brain stem at 24 hr. It seems that the effect on protein synthesis is transitory as no effect is visible after 4 hr. The increase in label in the brain stem 24 hr after precursor injection could also be due to a decrease in protein breakdown.

There is a large variability in the results even though the precaution of giving the labelled precursor by intraventricular injection was taken. This was probably due to errors in the injection technique and it was thought inappropriate to use this technique for subsequent experiments.

TABLE 4 : To show the effect of $ACTH_{4-10}$ treatment on the incorporation of ^{14}C leucine into protein labelled 1, 4, 24 and 48 hr after precursor injection.

Time after ^{14}C leucine	Treatment	Specific activity in acid insoluble fraction, d.p.m./mg protein		
		Hind brain	½ Cortex	Brain stem
1 hr	$ACTH_{4-10}$	1420±353(5)	806±421(10)	1780±437(5)
	Control	601±259(5)*	649±340(10)	918±319(5)**
4 hr	$ACTH_{4-10}$	1246±263(5)	823±248(10)	1307±895(5)
	Control	1311±208(5)	926±607(10)	1441±231(5)
24 hr	$ACTH_{4-10}$	583±123(5)	332±173(10)	578± 56(5)
	Control	458±281(5)	384±271(10)	261±139(5)*
48 hr	$ACTH_{4-10}$	470±210(5)	468±203(10)	559±238(5)
	Control	743±309(5)	433±137(10)	772±168(5)

Results as mean specific activity ± std. deviation (n)

* $p < 0.01$ (t-test)

** $p < 0.025$

TABLE 5 : To show the effect of $ACTH_{4-10}$ treatment on the relative specific activity of protein 1 and 4 hr after precursor injection.

Time after ^{14}C leucine	Treatment	Relative specific activity d.p.m./mg protein/ASF d.p.m.		
		Hind brain	Cortex	Brain stem
1 hr	$ACTH_{4-10}$	619.0±273.6(5)	333.4±101.1(10)	441.0±132.3(5)
	Control	265.8± 63.2(5)*	219.3± 65.6(10)	242.4±103.5(5)*
4 hr	$ACTH_{4-10}$	448.4±115.5(5)	324.3± 95.4(10)	325.2±195.3(5)
	Control	712.6±368.1(5)	427.3±274.9(10)	476.2±332.9(5)

Result as mean relative specific activity ± std. deviation (n)

* $p < 0.05$ (t-test)

(iv) To show the effect of $ACTH_{4-10}$ on rat brain protein metabolism using an intraperitoneal injection of ^{14}C-leucine

This experiment was designed to confirm the results of experiment 2 (iii) using intraperitoneal injection of precursor. Because of the practical difficulties in handling large numbers of rats, groups of animals were killed after only 3 different incorporation times. 2 hr, 24 hr and 6 days. In this way the groups could be large enough to allow statistical analysis of the results.

Method

30 male, albino Wistar rats were randomly assigned to one of 2 groups.

$ACTH_{4-10}$ group received daily intraperitoneal injections of a neutral solution of 20 µg. $ACTH_{4-10}$ in 0.2 ml physiological saline.

CONTROL Group received daily intraperitoneal injections of 0.2 ml saline.

On the 14th day of the experiment, all rats received an intraperitoneal injection of 5 µCi ^{14}C-L-leucine in 0.2 ml saline. Groups of rats were killed 2 hr, 24 hr and 6 days later. Samples of cerebellum, cortex, brain stem and liver were taken and carbohydrate and lipid-free protein was extracted and estimated as before. (Section 1 (iii) and the Appendix).

Results

There is no significant difference between the specific activity of protein of $ACTH_{4-10}$ treated and control rats (table 6). If the relative specific activity after 2 hr is considered (table 7) $ACTH_{4-10}$ treatment appears to stimulate the incorporation of leucine into protein.

Discussion

$ACTH_{4-10}$ increases the incorporation of ^{14}C-leucine into brain stem protein after a 2 hr incoporation time. This is seen when the relative specific activity is considered (table 9). That is, differences are seen when corrections are made for the level of label in the acid soluble fraction. This corrects for any differences in uptake of amino acid into the brain or errors in injection. The possibility that $ACTH_{4-10}$ causes an increase in the free pool of amino acids will be considered in Section 4.

TABLE 6 : To show the effect of $ACTH_{4-10}$ treatment on the incorporation of ^{14}C-leucine into brain and liver protein. Results are expressed as specific activity.

Group	Time after injection of ^{14}C-leucine 2 hr	24 hr	6 day
CEREBELLUM			
$ACTH_{4-10}$	217.6± 8.11(5)	200.8±27.4(5)	188 ±19.1(5)
Control	206.3±26.7 (5)	254.8±39.8(5)	186.6±24.6(5)
CORTEX			
$ACTH_{4-10}$	194.4±18.9(5)	221.4±36.2(5)	198.0±22.9(5)
Control	199.0± 8.6(5)	211.8±33.8(5)	195.0±10.5(5)
BRAIN STEM			
$ACTH_{4-10}$	188.3±10.9(5)	228.2±37.5(5)	197.3±22.9(5)
Control	197.0± 4.5(5)	225.5±36.9(5)	189.2±19.9(5)
LIVER			
$ACTH_{4-10}$	845.6±79.8(5)	676.0±112.0(5)	263.8±80.6(5)
Control	716.8±107.5(5)	627.2±135.1(5)	240.2±109.1(5)

Results expressed as mean specific activity ± std.deviation(n)

TABLE 7 : To show the effect of $ACTH_{4-10}$ treatment on the incorporation of ^{14}C-leucine into brain and liver protein. Results expressed as relative specific activity.

Group	Relative specific activity 2 hr after injection of ^{14}C-leucine		
	CEREBELLUM	BRAIN STEM	
$ACTH_{4-10}$	314.2 ± 89.6(5)N.S.	251.8 ± 41.3(5)	$p < 0.025$
Control	276.1 ± 77.2(5)	150.3 ± 57.8(5)	
	CORTEX	LIVER	
$ACTH_{4-10}$	254.8 ± 67.9(5)N.S.	420.8 ± 69.7(5)N.S.	
Control	181.1 ± 80.4(5)	319.4± 91.0(5)	

Results expressed as mean relative specific activity ± std.dev.(n)
Significance by student's t-test. N.S. = not significant

(v) To study the effect of $ACTH_{4-10}$ treatment on the incorporation of ^{14}C-leucine into brain protein

$ACTH_{4-10}$ increases the incorporation of ^{14}C-leucine into brain stem protein after 1 and 2 hr incorporation times (Section 2 (iii) and (iv)). It was of interest to investigate whether $ACTH_{4-10}$ produced changes in protein metabolism after a wide range of incorporation times or whether the effect was only maintained 1-2 hrs after injection of ^{14}C-leucine.

Method

Because of the practical difficulties of handling large numbers of animals, it was necessary to carry out a series of experiments to study the time course of the effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into brain protein.

The design of all experiments was the same. Male, albino Wistar rats weighing 140-160 gm were treated with either $ACTH_{4-10}$ or saline for 2 weeks. On the 14th day all rats received an IP injection of 5μCi ^{14}C-leucine. Groups of rats were then killed by decapitation and the brain divided into 3 gross areas: hind brain, cortex and brain stem. In most cases a sample of liver was taken as a control. Protein was extracted and estimated as described before (Section 1 (iii) and the Appendix).

Results

Although the design and conditions of all experiments was the same the absolute value of the specific activity

(dpm/mg protein) varied considerably between experiments. If the results are expressed as mean specific activity of all experiments (fig. 1-4) it may be seen that there is a tendency for $ACTH_{4-10}$ to increase the incorporation of ^{14}C-leucine into hind brain and brain stem protein between 1 and 4 hr after precursor injection. Unfortunately, as the standard deviation is excessively large (approx. 50%) these differences are not significant. Other workers have found similar discrepancies between experiments of this nature (Rose, 1967; Bateson et al., 1969; Talwar et al., 1966) and express their results in terms of a corrected mean. The acid soluble fraction gives an indication of the free pool of amino acids and of the efficacy of the precursor injection. A correction was made for this by expressing the results as relative specific activity. This correction is not possible for times after 4 hr because the ASF values are very low. To correct for differences between experiments, these results were expressed as a percentage of the total mean in each experiment.

The results expressed in this manner are given in table 8 and fig. 5-7. It may be seen that the incorporation of leucine into protein is increased in the brain stem and hind brain from rats treated with $ACTH_{4-10}$. This effect is first apparent 1 hr after injection of leucine.

Fig. 1 : The effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into rat hind brain protein, in vivo.

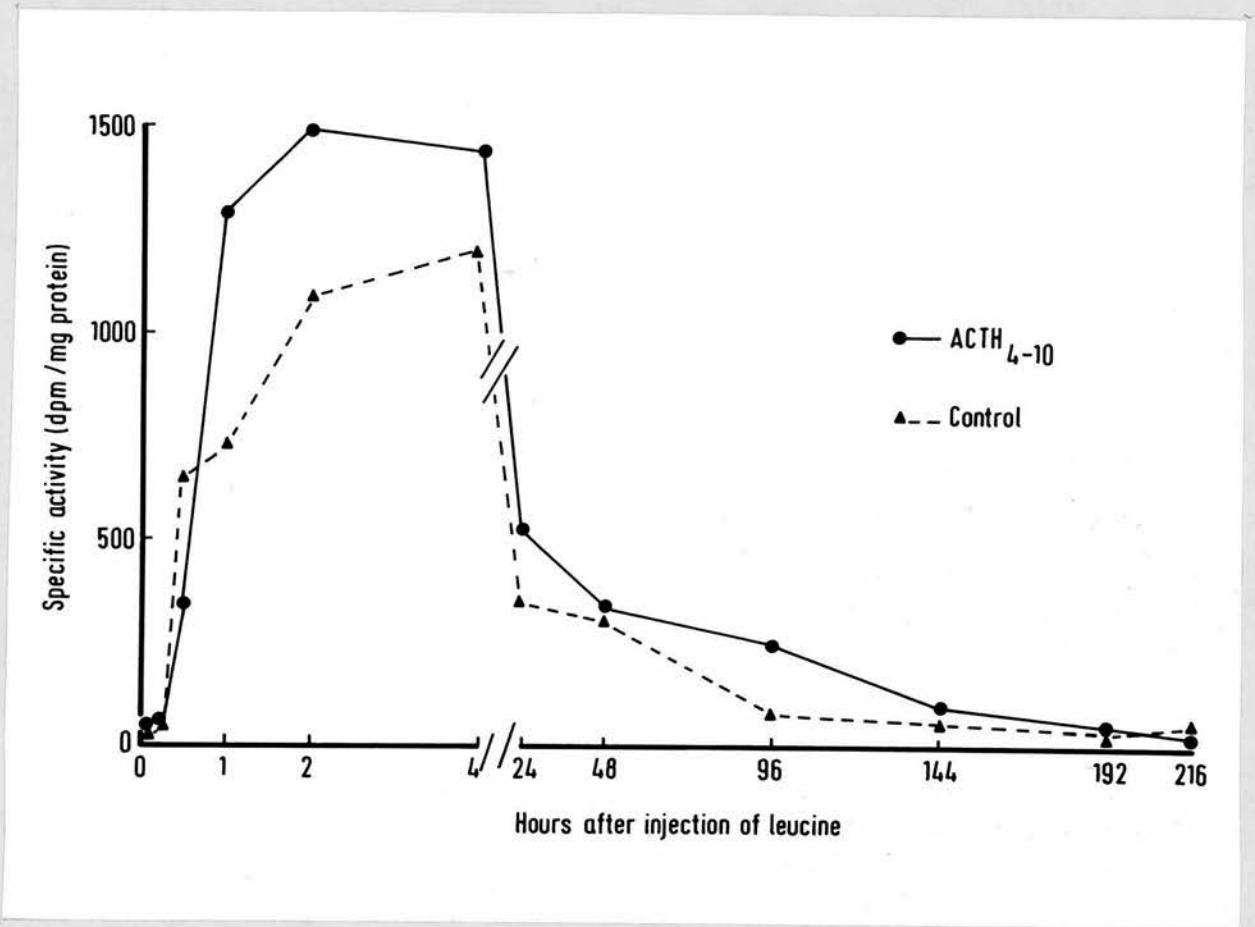


Fig. 2 : The effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into rat brain cortex protein, in vivo

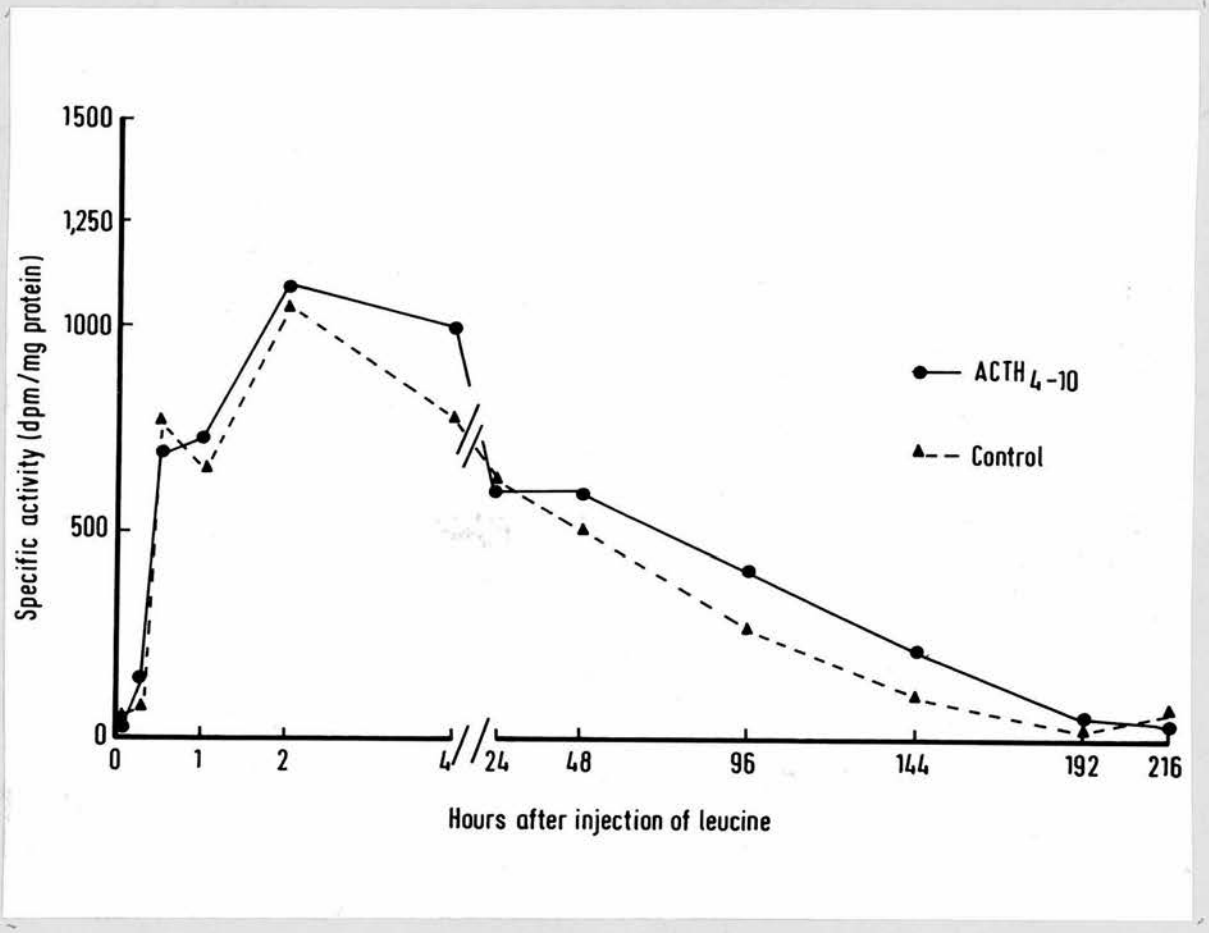


Fig. 3 : The effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into rat brain stem protein, in vivo.

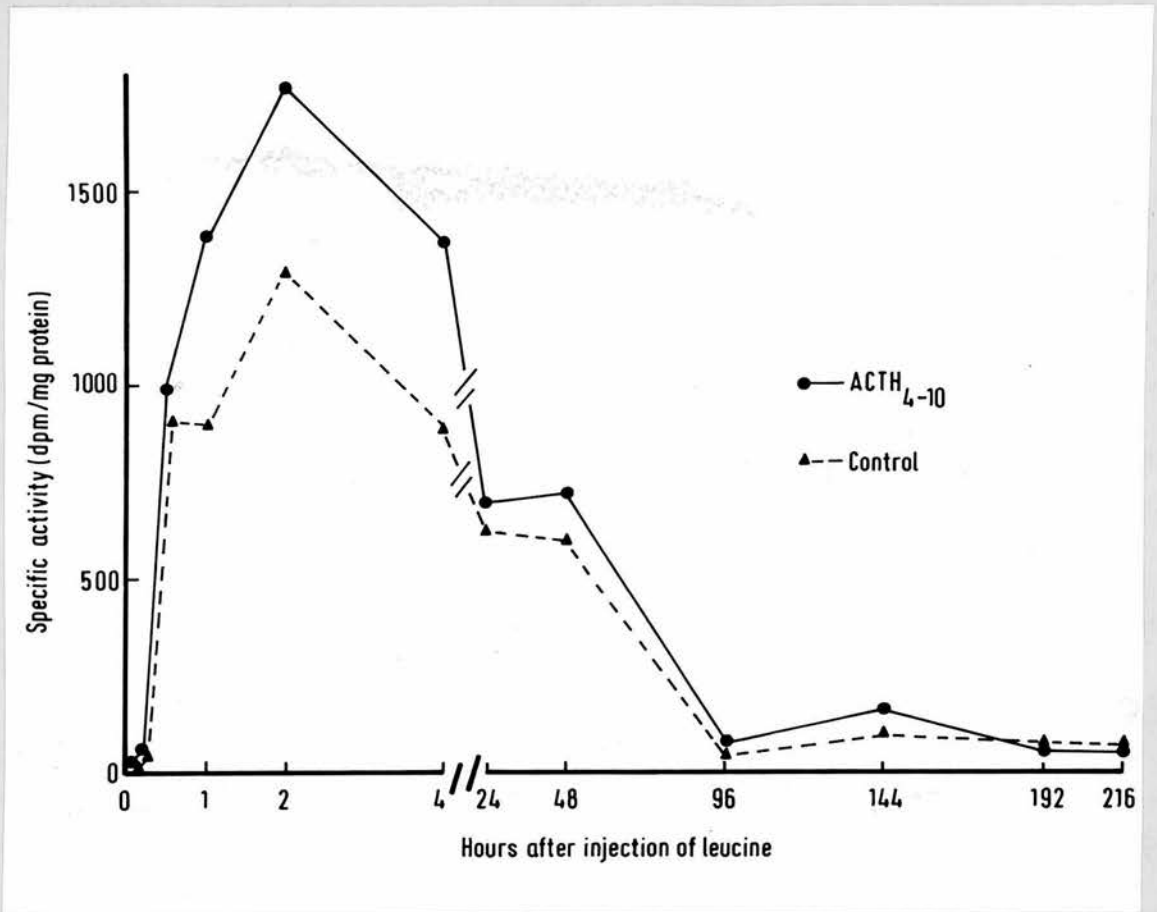


Fig. 4 : The effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into rat liver protein, in vivo.

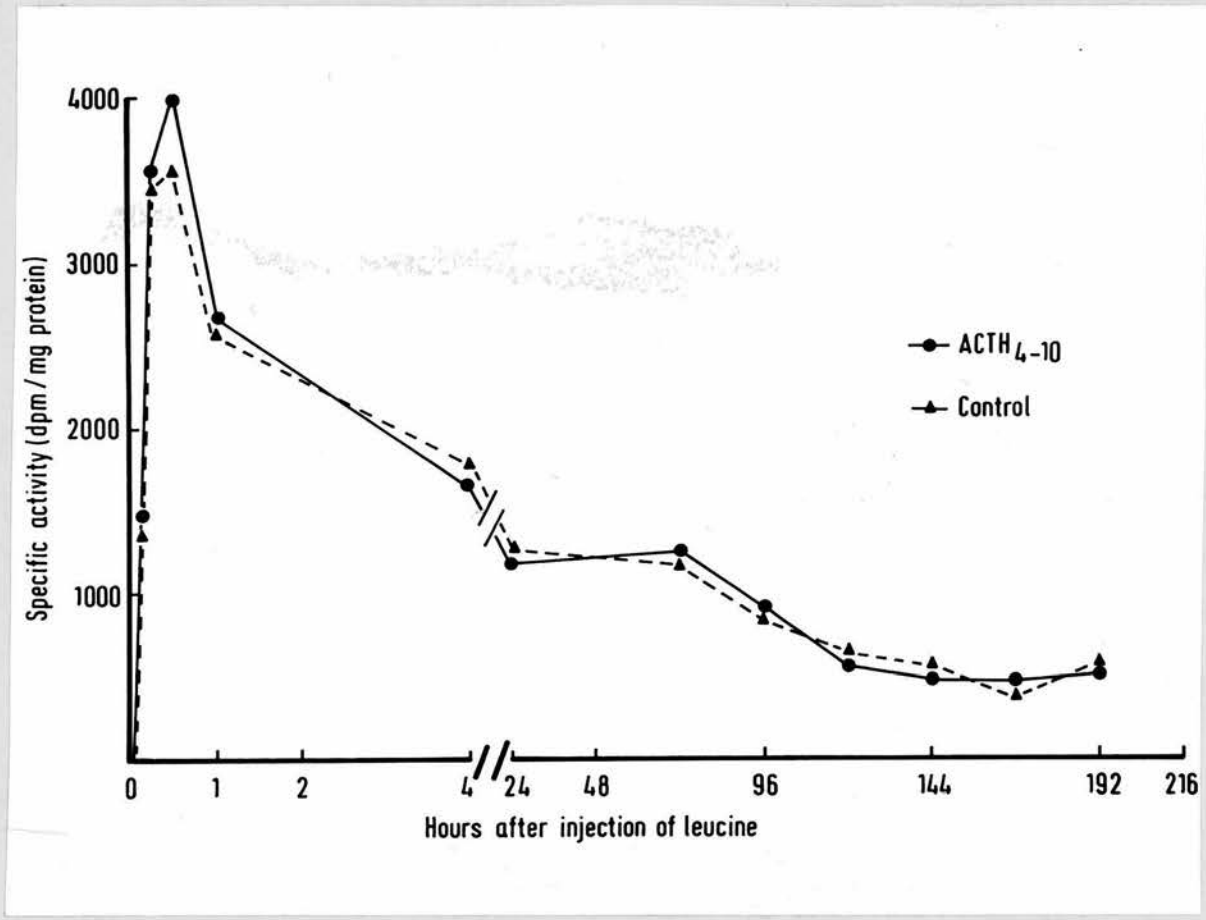


TABLE 8 : To show the effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into brain protein, in vivo.

Time	Treatment	Brain Area: Hind Brain	Cortex	Brain Stem
5 min	$ACTH_{4-10}$	99.67 ± 25.2(11)N.S.	96.2 ± 88.4(11)N.S.	104.8 ± 17.1(11)N.S.
	Control	100.55 ± 32.3(11)	103.7 ± 23.9(11)	95.2 ± 28.3(11)
15 min	$ACTH_{4-10}$	102.9 ± 45.7(6) N.S.	117.9 ± 31.6(6) N.S.	84.8 ± 25.4(6) N.S.
	Control	97.5 ± 72.8(6)	82.1 ± 36.4(6)	115.2 ± 79.1(6)
30 min	$ACTH_{4-10}$	132.1 ± 54.8(5) N.S.	103.9 ± 48.2(6) N.S.	104.0 ± 27.1(6) N.S.
	Control	68.6 ± 51.2(5)	96.0 ± 61.7(6)	96.0 ± 56.6(6)
60 min	$ACTH_{4-10}$	131.1 ± 48.8(20)t=4.16	99.2 ± 38.9(20)N.S.	120.1 ± 45.8(20)t=3.0
	Control	69.1 ± 42.9(20)P < .001	100.8 ± 47.7(20)	79.9 ± 36.4(20)P < .01
120 min	$ACTH_{4-10}$	106.4 ± 71.8(5) N.S.	116.9 ± 34.8(5) N.S.	128.8 ± 24.4(5)t=3.59
	Control	93.5 ± 82.5(5)	83.1 ± 53.4(5)	71.9 ± 20.3(5)P < .01
240 min	$ACTH_{4-10}$	111.7 ± 56.1(21)N.S.	107.2 ± 32.4(21)N.S.	126.2 ± 43.9(21)t=3.7
	Control	88.2 ± 54.2(21)	92.7 ± 64.2(21)	73.3 ± 46.5(21)P < .001

Results expressed as mean (% total corrected mean in each experiment) ± standard deviation.
Number of animals in brackets. N.S. = not significant
The Students t-test was used for the statistical analysis of the results.

Fig. 5 : The effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into rat hind brain protein, in vivo.

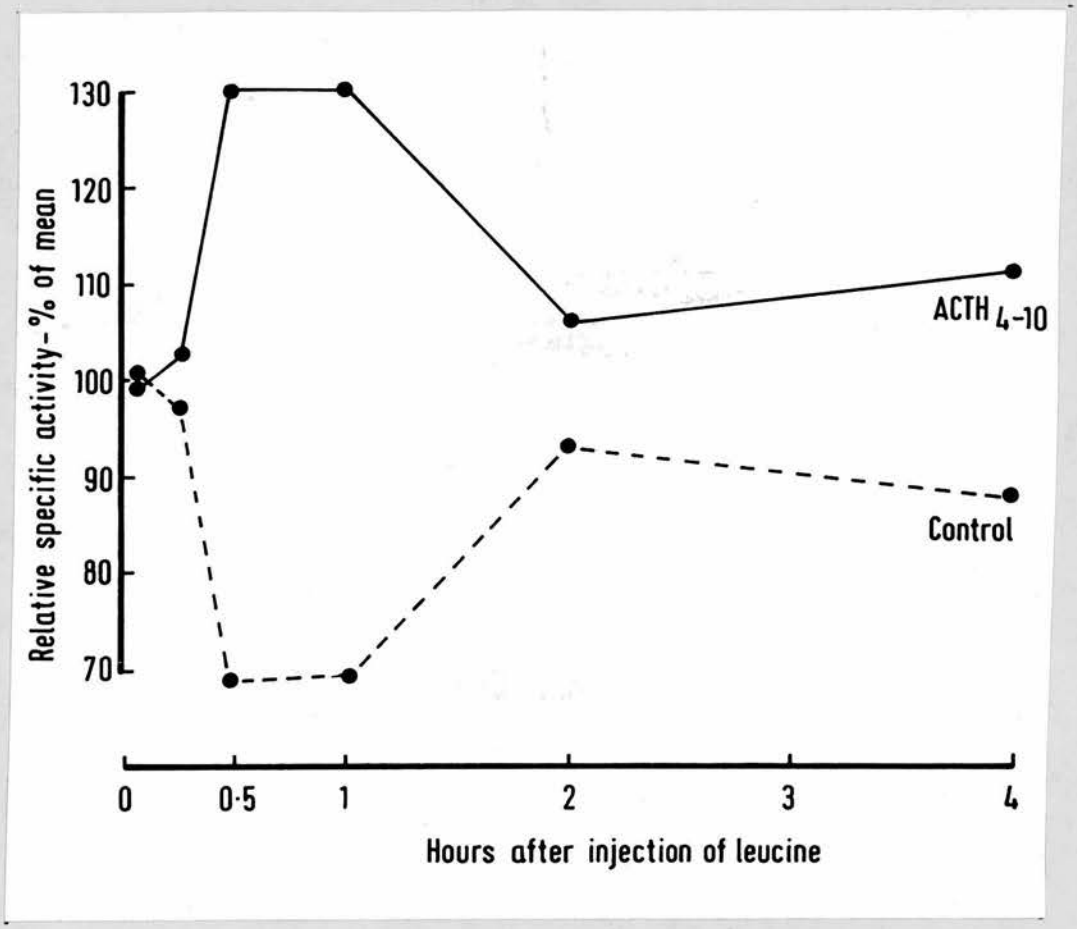


Fig. 6 : The effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into rat brain cortex protein, in vivo.

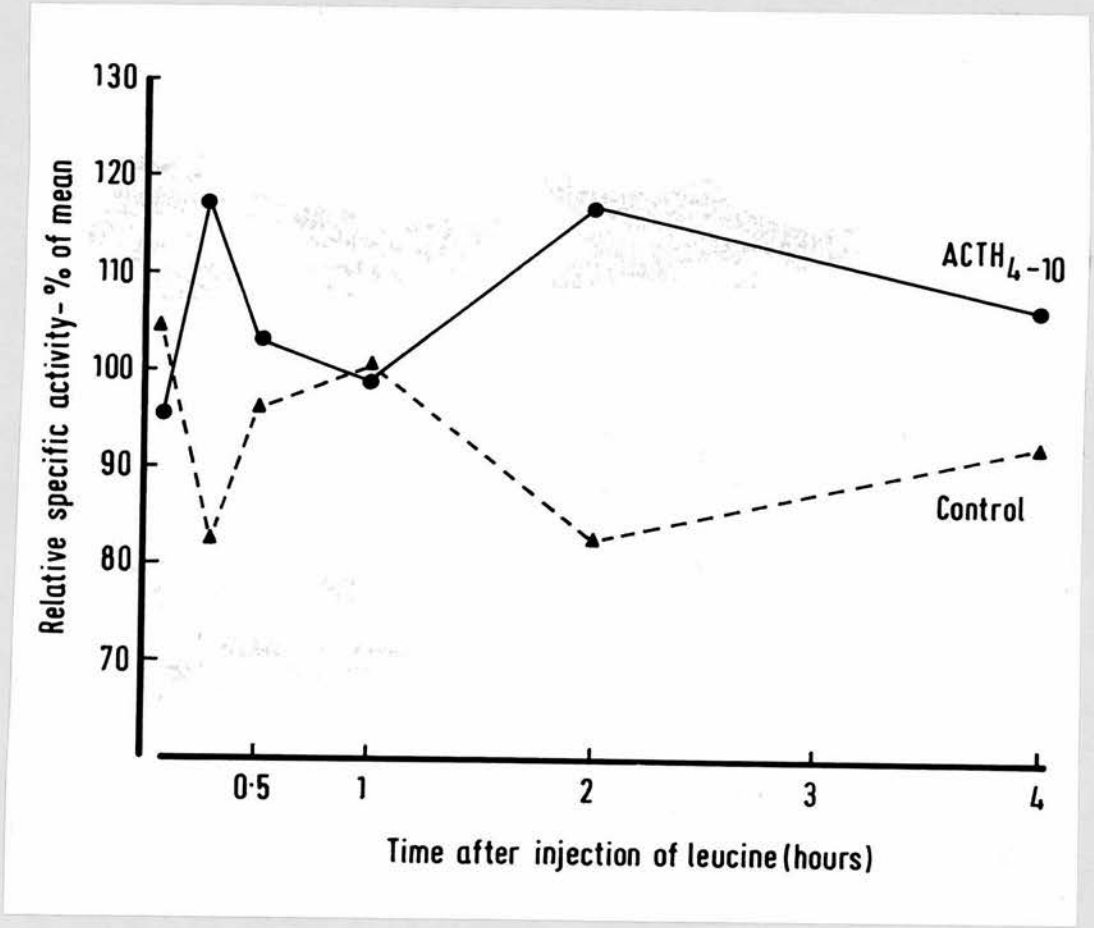
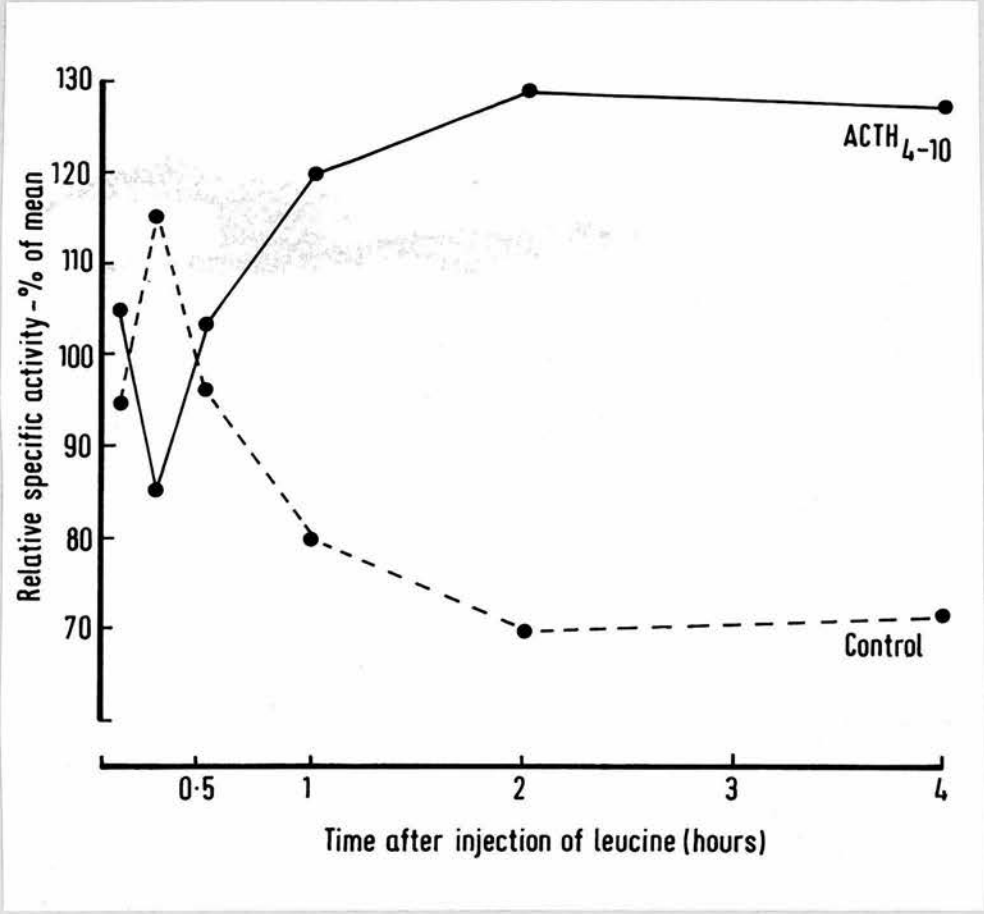


Fig. 7 : The effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into rat brain stem protein, in vivo.



Discussion

$ACTH_{4-10}$ increases the incorporation of ^{14}C-leucine into the protein of the brain stem and hind brain. There is no effect in the cortex and liver. The change in the brain stem is first seen 1 hr after precursor injection and is maintained for the further 3 hr studied. The increase in the hind brain also appears after 1 hr but is not maintained. There is a tendency for an increase in incorporation in the hind brain 30 min after leucine injection but this is not significant.

(vi) To study the effect of $ACTH_{4-10}$-7-D-phe treatment on the incorporation of ^{14}C-leucine into brain protein

$ACTH_{1-10}$-7-D-phe effects behaviour in the opposite way to the all-L isomer, $ACTH_{1-10}$. $ACTH_{1-10}$-7-D-phe treatment deteriorates the acquisition of a conditioned avoidance response in the hypophysectomized rat and facilitates extinction in the intact rat, while $ACTH_{1-10}$ has the opposite effect (Bohus and de Wied, 1966; de Wied, 1969). $ACTH_{1-10}$-7-D-phe has also been shown to have the opposite effect to the all-L peptide in the biochemical studies on the hypophysectomized rat. It reduces the incorporation of ^{14}C-leucine into rapidly turning over brain stem protein (Schotman et al., 1972).

It was of interest to see if $ACTH_{4-10}$-7-D-phe had any effect on the incorporation of ^{14}C-leucine into brain protein of the intact rat.

Method

The experimental design was similar to that in the previous experiment. Albino Wistar rats, weighing 140-160 gm were randomly assigned to one of two groups.

$ACTH_{4-10}$-7-D-phe group which received daily intraperitoneal injections of 0.2 ml of a neutral solution containing 100 µg/ml peptide in physiological saline.

CONTROL group which received daily intraperitoneal injections of 0.2 ml of physiological saline.

On the 14th day of the experiment all rats received an intraperitoneal injection of 5µCi ^{14}C-L-leucine in 0.2 ml saline. Groups of rats were killed by decapitation 30 min, 1 hr and 4 hr after injection of leucine. The brain was divided into 3 areas; hind brain, brain stem and cortex. Carbohydrate and lipid-free protein was extracted and estimated as described before (Section 1 (iii) and the Appendix.).

Results

The results are expressed as a percentage of the mean of the total relative specific activity in each experiment - as in the previous experiment (Section 2 (vi)). It may be seen from table 9 that $ACTH_{4-10}$-7-D-phe treatment tends to reduce the amount of labelled protein synthesized in the brain stem /hind brain in the first 30 min after precursor injection and also reduces the incorporation into the brain stem after 4 hr.

TABLE 9 : To show the effect of $ACTH_{4-10}$-7-D-phe on the incorporation of ^{14}C-leucine into brain protein in vivo.

Time	Treatment	Brain Area: Hind Brain	Cortex	Brain Stem
30 min	Peptide	85.8 ± 5.2(3) t = 4.81	118.6 ± 19.3(3)	95.2 ± 3.6(3) t = 2.9
	Control	116.3 ± 7.3(3) P < 0.01	72.0 ± 18.3(3) N.S.	105.0 ± 3.11(3) P < 0.05
60 min	Peptide	105.5 ± 28.5(5) N.S.	87.7 ± 23.8(5) N.S.	93.0 ± 17.6(5) N.S.
	Control	95.2 ± 31.2(5)	113.1 ± 27.5(5)	107.0 ± 14.9(5)
240 min	Peptide	94.4 ± 17.7(6) N.S.	113.2 ± 27.5(6) N.S.	85.1 ± 14.9(6) t = 2.6
	Control	106.9 ± 18.6(6)	87.9 ± 22.7(6)	114.3 ± 19.9(6) P < .05

Results expressed as mean per cent relative specific activity ± standard deviation (n)

The Students t-test was used for the statistical analysis of the results.

N.S. = not significant.

Discussion

$ACTH_{4-10}$-7-D-phe tends to have the opposite effect to $ACTH_{4-10}$ on the incorporation of leucine into protein of the intact rat. The pattern of results in this experiment is very similar to that in the previous experiment (Section 2 (v)) in that the peptide produces its effects in the hind brain and in the brain stem while the cortex remains unaffected. Only the changes in the brain stem are maintained. It should be noted that the number of animals in each group, especially after 30 min, is very small. Unfortunately time did not permit as large a number of rats to be investigated as in the $ACTH_{4-10}$ studies.

(vii) General Discussion

$ACTH_{4-10}$ treatment increased (Section 1 (v)) and $ACTH_{4-10}$-7-D-phe treatment tends to decrease (Section 1(vi)) the incorporation of ^{14}C-leucine into brain protein. These effects are seen 0.5 to 1 hr. after precursor injection. Although changes are seen in both the hind brain and brain stem areas they are only maintained in the brain stem. Peptide treatment has no effect on protein metabolism in the cortex.

These results may be compared with those of Schotman (Schotman et al., 1972) who found that in hypophysectomized rats $ACTH_{1-10}$ treatment stimulated the synthesis of brain stem proteins with a short half-life. This was not found in intact rats. $ACTH_{4-10}$ treatment had no effect 5 and 15 min after precursor injection.

There could be several explanations for these differences in response of intact and hypophysectomized rats. It is possible that we are looking at 2 completely different responses in the 2 models; in the hypophysectomized rat ACTH peptides are effecting proteins with a short half-life and, in the intact rat, proteins with a longer half life. Although the hypophysectomized animal is subject to serious metabolic derangements caused by the hormonal imbalance it seems unlikely, although not impossible that it would respond by a different mechanism to the intact rat. A more probable explanation for the differences is that the hypophysectomized rat is more sensitive to the action of the peptides than the intact rat.

After hypophysectomy, brain protein synthesis is reduced (Dunn and Korner, 1966; Takahshi et al., 1970). One might expect that it would be rather more easy to increase reduced protein synthesis than upset the normal homeostatic mechanisms found in the intact rat. If this is true the changes seen in the hypophysectomized rat would be larger and therefore more easily detected than in the intact rat. It is likely that the changes in the intact rat would only be visible at the time of maximum incorporation (1-4 hr), the times when, in fact, increases in incorporation were detected in the brain stem.

$ACTH_{4-10}$ could increase the incorporation of ^{14}C-leucine into brain stem proteins by various mechanisms:-

1. By modifying nucleic acid metabolism. As ACTH peptide treatment has no effect on polysome profiles (Gispen

and Schotman, 1970; Gispen et al., 1971), on RNA synthesis (Schotman et al., 1972; Reading and Dewar, 1971) or on RNA polymerase activity (Dewar, 1971), it seems unlikely that the peptides act by this mechanism.

2. By reducing protein breakdown. This would explain the increase in labelled protein 1 hr after precursor injection seen in the intact rat but it has too long a time course toexplain the effects on rapidly turning-over protein seen in the hypophysectomized rat.

3. By increasing the pool of free amino acids in the brain by either mobilizing amino acids from a source in the body or by increasing the transport of amino acids to some site in the brain.

4. By an effect on the protein synthetic mechanism at the translation level. As ACTH peptide analogues increase incorporation of leucine into protein while having no effect on nucleic acid metabolism, this is a possible mechanism of action.

The alternative possible modes of action of ACTH peptides will be discussed further in sections 4 and 5.

SECTION 3

TO SHOW THE EFFECT OF $ACTH_{4-10}$ TREATMENT ON THE INCORPORATION OF ^{14}C-LEUCINE INTO THE PROTEIN OF VARIOUS SUBCELLULAR FRACTIONS OF BRAIN TISSUE.

(i) Introduction

It has been shown in section 2 that $ACTH_{4-10}$ increases the incorporation of ^{14}C-leucine into brain protein. It was of interest to discover whether the extra protein was available for general use in the cell or was found mainly in one brain cell organelle. The fate of the protein might give us some information on the mechanism of action and significance of peptide action.

Various procedures have been reported to alter the incorporation of leucine into brain tissue subcellular fractions. For example, Kuschinsky and Vogt (1971) found a rise in protein content of curde myelin-microsomal fraction of mouse brain with a decreased incorporation of ^{14}C leucine into synaptosomal protein after morphine treatment. Treatment of mice brain with barbital slightly inhibits the uptake of ^{14}C-leucine into synaptosomal protein while markedly decreasing the uptake into mitochondrial protein (Kuschinsky, 1971).

Using subcellular fractionation techniques, Schotman et al. (1972) concluded that up to 5 min. after injection of leucine into hypophysectomized rats most of the acid insoluble radioactivity is located in growing peptide chains attached to polysomes bound to nuclear membranes to free polysomes in the cytoplasm and to insoluble cytoplasmic protein. At this time animals treated with

$ACTH_{1-10}$ had an increased incorporation of leucine into brain protein. In the intact rat, it has been reported that $ACTH_{4-10}$ treatment increases the incorporation of ^{14}C-leucine into synaptosomal protein 24 hr. after precursor injection, while having no effect at other times or in other fractions (Reading, personal communication).

As only a small proportion of labelled protein is found in the synaptosomal fraction changes in this fraction would not account for the marked changes seen in whole brain. One might also expect to see changes in the microsomal and supernatant fractions prior to the changes in the synaptosomes. In view of the evidence that $ACTH_{4-10}$ increases the incorporation of ^{14}C-leucine into whole brain protein, 1-4 hr. after leucine injection (Section 2) these results are rather difficult to interpret.

The effect of $ACTH_{4-10}$ treatment on the incorporation of ^{14}C-leucine into the protein of various subcellular fractions of brain tissue was investigated.

(ii) General Method

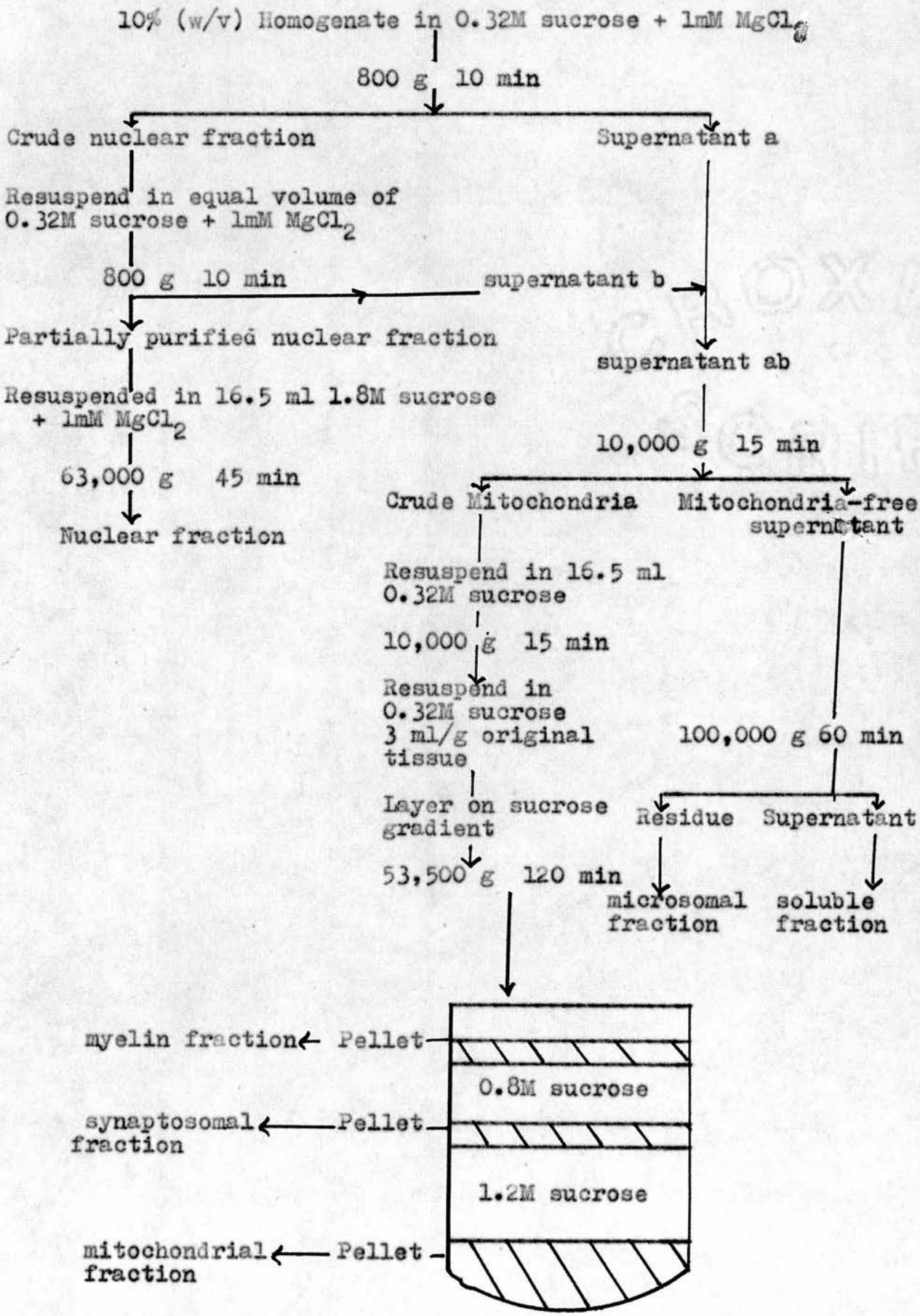
The method of subcellular fractionation devised was based on that used by Whittaker for preparing synaptosomes (Whittaker and Barker, 1972). This method had to be modified so that all subcellular fractions, including nuclei could be prepared satisfactorily. Brain nuclei, especially neuronal nuclei are fragile, so a compromise had to be made to obtain the maximum yield of synaptosomes with the minimum amount of damage to the nuclei. The clearance of the homogenizer was increased from 4 thou. to

7 thou. to reduce mechanical damage to the nuclei. Divalent cations are essential for the preservation of nuclear structure (Hogeboom et al., 1952) but cations can cause aggregation of the myelin fraction, changing the sedementation properties of this fraction. Calcium appears to be more active in this latter effect than magnesium, consequently 1 mM - $MgCl_2$ was added to the 0.32 M - sucrose homogenization medium.

A flow diagram of the method used is given in fig. 1. All procedures were carried out at 0-4°C. Whittaker recommends that all equipment used should be precooled and then all procedures carried out in a cold room (Whittaker and Barker, 1972). Unfortunately cold room facilities were not available. All equipment and rotors were precooled to 0-4°C but the experiment took place at room temperature. Where ever possible operations were carried out in a Lager cooler and solutions and equipment were kept in ice baths.

Male, albino Wistar rats weighing 140-160 gm were used throughout. Each rat was killed by decapitation and the brain removed as quickly as possible. This was dissected into 2 brain areas, brain stem and cortex, in a Lager cooler. These samples were weighed and homcgenized in 10 vol. 0.32 M sucrose plus 1 mM $MgCl_2$ for 2 min at 960 rev/min. Great care was taken to keep the mixture cool during homogenization. The homogenate was centrifuged at 800 g for 10 min in a Mistral 2L (MSE) centrifuge. The pellet was washed in sucrose and the supernatants

Fig. 1 : Flow diagram of the method of subcellular fractionation of brain tissue



pooled. The pellet, the partially purified nuclear fraction, was resuspended in 16.5 ml of 1.8 M sucrose with 1 mM $MgCl_2$ and spun at 63,000 g for 45 min in a Beckman Model L2 65B ultracentrifuge giving the nuclear fraction (Dewar, 1972). At the same time the pooled supernatants were spun for 15 min at 10,000 g in an MSE Superspeed 65 ultracentrifuge. The supernatant was reserved and the pellet, the crude metochondrial fraction, was washed with 0.32 M sucrose. The pellet was resuspended in 3 ml 0.32 M sucrose for each gram original wet weight of tissue. (There was approx. 0.5 gm wet weight). This suspension was carefully layered on a discontinueous gradient of 0.8 M and 1.2 M sucrose. This was centrifuged at 53,500 g for 120 min in the MSE, ultracentrifuge giving layers of myelin, synaptosomes and mitochondria. The pooled supernatants formed in the preparation of the crude mitochondrial fraction were spun at 100,000 g for 60 min on the Beckman ultracentrifuge after the preparation of the nuclei. This gave a pellet of microsomes and a supernatant containing soluble proteins.

All the fractions prepared as above were transferred to Eppendorf tubes where protein precipitation was carried out. Ten volumes 10% TCA was added to all the fractions formed as a pellet i.e. nuclei, mitochondria and microsomes. 50% TCA, to give a final concentration of 10% was added to the fractions formed in sucrose suspension i.e. myelin, synaptosomes and soluble fraction. All fractions were kept in ice for 10 min to allow complete

precipitation of protein. They were then centrifuged in an Eppendorf microcentrifuge and washed at least twice with 10% TCA. Lipid and nucleic acid free protein was extracted and estimated as described in section 1 (iv).

(iii) Purity of fractions

Purity of subcellular fractions may be estimated by use of the electron microscope or by study of enzyme markers. Electron microscopy is the method of preference as smallsamples of each fraction can give an indication of purity. Use of enzyme markers is rather more problematic as frequently enzymes are found in more than one fraction. For example fumarase is used as a mitochondrial marker, lactate dehydrogenase for soluble cytoplasma and Na, K-activated ATPase for external membranes, but all three enzymes are found in synaptosomes which have cytoplasm with glycolytic enzymes and small mitochondria enclosed within an external membrane. Unfortunately electron microscope facilities were not available making it necessary to use enzyme markers. The enzymes selected were $Na,^{+}K^{+}$-ATPase, Mg^{2+}ATPase, fumarase and lactate dehydrogenase. The methods of estimation of these enzymes are given in the Appendix. Enzyme activities in each fraction of a typical separation are given in table 1. Lactate dehydrogenase is a soluble cytoplasmic enzyme and is found primarily in the supernatant fraction but is also found in the synaptosomes (Johnson and Whittaker, 1963). The results given in table 1 also indicate enzyme activity in the microsomal fraction suggesting that there was some

TABLE 1 : To show the distribution of various enzymes in subcellular fractions of brain tissue prepared as described in Section 3 (ii).

Fraction	Lactate Dehydrogenase (uMole NADPH oxidized/mg protein/ min)	Fumarase (ΔE/mg protein/ 5 min)	Na^+, K^+-ATPase (uMole P_i liberated/ mg protein/hr.)	Mg^+, ATPase (uMole P_i liberated/ mg protein/hr.)
Total Homogenate	13.28 ± 0.19	0.443 ± 0.091	11.45 ± 0.94	9.66 ± 1.03
Impure Mitochondria	2.25 ± 0.11	0.41 ± 0.085	6.99 ± 0.51	4.31 ± 0.49
Nuclear Debris	0.18 ± 0.03	0.004 ± 0.001	4.62 ± 0.52	5.36 ± 0.36
Nuclei	0.005 ± 0.001	0.0 ND -	7.64 ± 2.14	13.53 ± 1.96
Myelin	0.08 ± 0.012	0.31 ± 0.076	7.02 ± 0.49	6.54 ± 0.21
Synaptosomes	1.7 ± 0.31	1.83 ± 0.109	24.36 ± 2.01	17.18 ± 0.52
Mitochondria	0.167 ± 0.03	2.24 ± 0.112	12.66 ± 0.21	6.46 ± 0.64
Microsomes	0.288 ± 0.091	0.086 ± 0.010	5.94 ± 0.51	4.20 ± 0.21
Supernatant	6.0 ± 0.14	0.009 ± 0.001	ND	ND

The results represent the mean of 3 experiments ± standard deviation.

(ND = none detected)

contamination of this fraction. Fumarase is an enzyme of the citric acid cycle which is found in mitochondrial fraction. Activity is also found in the synaptosomal fraction due to the presence of small mitochondria inside the synaptosomes (Whittaker and Barker, 1972). This pattern may be seen in the results presented in table 1. In addition the myelin fraction showed a low enzyme activity suggesting the presence of some small mitochondrial particles. ATPases are membrane markers and are found in all fractions except the supernatant fraction Na^+,K^+-ATPase is primarily found in the microsomal (Deul and McIlwain, 1961; Schwartz et al., 1962) and in the synaptosomal membranes (Kurokawa et al., 1965; Hosie, 1965) Na^+,K^+-ATPase activity was primarily associated with these fractions (table 1) but activity was also found in the myelin and mitochondrial fractions. Other workers have found a similar degree of contamination in these fractions (Kurokawa et al., 1965) Mg^{2+}ATPase was found in all fractions except the supernatant. These results suggest that the fractions prepared here were of a similar degree of purity to those prepared by other workers with the exception of the microsomal fraction which seems to contain a substantial amount of trapped cytosol.

(iv) <u>To show the effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into subcellular fractions of brain tissue</u>

(a) <u>Method</u>

Male, albino Wistar rats weighing 140-160 gm were

randomly assigned to one of two groups.

Control group received daily IP injections of 0.2 ml physiological saline

$ACTH_{4-10}$ group received daily IP injections of 20 ug $ACTH_{4-10}$ in 0.2 ml saline.

On the 14th day of the experiment all rats received an IP injection of 10 uCi ^{14}C-leucine. Groups of rats were killed at varying times after this injection and subcellular fractions from the cortex and brain stem (Gispen et al., 1972) were prepared as described above (Section 3 (ii)). Protein was extracted and estimated as in Section 1 (iii).

Because of the length of time required to prepare the subcellular fractions, few samples could be separated at one time. Consequently it was necessary to carry out a series of experiments to obtain sufficient results for statistical analysis. The experimental conditions were kept the same throughout. To correct for any differences between experiments, the results were expressed as a percentage of the total mean specific activity for each fraction. The specific activity is defined as the dpm/mg TCA unsoluble protein extracted from the fraction.

Groups of rats were killed, 15 min, 1 hr., 2 hr., 24 hr., 48 hr., 120 hr., 144 hr., and 168 hr. after the administration of ^{14}C-leucine.

(b) Results

If the results of just one experiment in the series are considered (table 2) the distribution of labelled leucine in the acid insoluble protein of the various fractions may

TABLE 2 : To show the effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into subcellular fractions of brain stem tissue in vivo.

Group	Time after administration of ^{14}C-leucine 2 hr.		24 hr.	
	Nuclei			
$ACTH_{4-10}$	324.5±23.4(3)	$P < 0.05$	212.8±31.2(3)	$P < 0.05$
CONTROL	262.1±19.7(3)		90.3±65.5(3)	
	Myelin			
$ACTH_{4-10}$	48.2± 7.1(3)	N.S.	72.8± 9.2(3)	N.S.
CONTROL	43.6± 7.1(3)		79.9± 3.7(3)	
	Synaptosomes			
$ACTH_{4-10}$	84.6±11.6(3)	N.S.	86.1± 9.0(3)	N.S.
CONTROL	66.2± 9.18(3)		75.7±25.3(3)	
	Mitochondria			
$ACTH_{4-10}$	109.9±38.8(3)	N.S.	74.0±24.8(3)	N.S.
CONTROL	106.3± 7.8(3)		117.6±15.0(3)	
	Microsomes			
$ACTH_{4-10}$	158.8±11.5(3)	N.S.	101.9±12.4(2)	$P < 0.05$
CONTROL	175.8±21.4(3)		56.5± 4.6(3)	
	Supernatant			
$ACTH_{4-10}$	208.9±21.5(3)	N.S.	113.3±39.2(2)	N.S.
CONTROL	173.5±12.0(3)		148.5±95.1(3)	

Results expressed as mean dpm/mg protein ± standard deviation
Number of animals in parenthesis
Significance by student's t-test with Bessel's correction.

be studied. Two hoursafter administration of leucine, the nuclear fraction shows the highest specific activity with the microsomal fraction having less than two thirds this value and the myelin fraction showing the lowest specific activity. After 24 hr. a similar pattern is seen although the differences in specific activity are not so great. The results shown in table 2 also indicate that $ACTH_{4-10}$ treatment stimulates the incorporation of leucine into the nuclear and microsomal fractions.

When the combined results of all experiments are considered (tables 3 and 4), a similar pattern may be seen. In the fractions prepared from the brain stem (table 3) there seems to be a tendency for $ACTH_{4-10}$ treatment to increase the incorporation of ^{14}C-leucine into the nuclear and microsomal fractions but this is not a consistant effect. For example, after 15 min or 60 min incorporation times $ACTH_{4-10}$ decreases incorporation of label into microsomal protein.

$ACTH_{4-10}$ treatment has no effect on the incorporation of ^{14}C-leucine into acid insoluble protein of fractions prepared from the cortex (table 3).

Discussion

$ACTH_{4-10}$ does not increase the incorporation of ^{14}C-leucine into the protein of any particular brain subcellular fraction. Slight increases and decreases in incorporation are seen in the nuclear and microsomal fractions, but these changes are not consistant. It seems

TABLE 3 : To show the effect of $ACTH_{4-10}$ treatment on the incorporation of ^{14}C-leucine into subcellular fractions of brain stem tissue.

Time (hr)	Group	Nuclei	Myelin	Synaptosomes	Mitochondria	Microsomes	Supernatant
.25	$ACTH_{4-10}$	81.9±41.2(3)	77.1±41.9(3)	81.1±44.9(3)	106.8±19.9(3)	72.9± 1.7(3)	
	CONTROL	118.1±53.2(3)	122.8±63.1(3)	118.8±50.4(3)	93.1±20.5(3)	116.2±18.3(3)*	
1	$ACTH_{4-10}$	103.9±33.8(6)	90.5±28.6(6)	112.7±40.9(6)	100.4±13.1(6)	85.0±13.7(6)	109.3± 9.7(4)
	CONTROL	96.1±26.9(6)	110.6±24.2(6)	87.3±40.8(6)	99.5±28.7(6)	115.0±14.3(6)ø	90.7±10.9(4)
2	$ACTH_{4-10}$	112.9±75.3(7)	95.4±20.6(7)	97.4±37.4(6)	93.2±29.7(5)	101.3± 9.7(5)	109.3±11.3(3)
	CONTROL	87.1±28.9(7)	104.7±15.6(7)	102.6±17.6(6)	106.8±11.9(5)	98.7±12.9(5)	90.7± 5.51(3)
24	$ACTH_{4-10}$	126.8±22.2(7)	90.8± 9.7(5)	89.4±15.7(5)	66.4±21.6(5)	119.6±15.0(5)	75.7±21.7(3)
	CONTROL	73.2±28.9(7)ø	109.2±11.8(5)	110.6±33.1(5)	124.4±47.6(5)	80.4±17.1(5)ø	124.4±11.0(3)*
48	$ACTH_{4-10}$	108.0±36.5(4)	88.3±14.0(4)	78.6±29.4(4)	117.9±21.6(4)	100.7± 6.4(4)	102.9±10.6(4)
	CONTROL	93.1±31.9(4)	111.7±17.0(4)	121.3±44.4(4)	82.1±26.7(4)	99.3± 6.4(4)	97.2± 8.3(4)
120	$ACTH_{4-10}$	95.0± 8.3(4)	101.0±23.3(4)	80.5±15.6(4)	94.0±10.6(4)	110.7± 9.0(4)	87.4±19.8(4)
	CONTROL	104.9±14.1(4)	99.0±17.5(4)	119.6±15.5(4)*	106.0± 9.0(4)	91.5±14.2(4)*	112.6±11.3(4)
144	$ACTH_{4-10}$	105.5±14.8(4)	90.8± 6.7(4)	93.5±17.5(4)	91.3± 6.0(4)	97.2± 9.6(4)	91.4± 7.6(4)
	CONTROL	94.6± 6.4(4)	109.2±11.7(4)	106.5±22.1(4)	108.7± 6.6(4)	102.8± 4.7(4)	108.7± 4.4(4)**
168	$ACTH_{4-10}$	116.3±28.5(4)	94.4±19.9(4)	112.9±12.9(4)	100.7±16.6(4)	101.2±18.7(4)	
	CONTROL	83.7±13.8(4)	105.6±15.4(4)	86.3±20.7(4)	99.4±16.5(4)	98.8±15.7(4)	

Results expressed as mean % specific activity ± standard deviation (n)

* $p < 0.05$
** $p < 0.02$
ø $p < 0.01$

The students t-test with Bessel's correction was used for the statistical analysis of the results.

TABLE 4 : To show the effect of $ACTH_{4-10}$ treatment on the incorporation of ^{14}C-leucine into subcellular fractions of brain cortex tissue.

Time (hr)	Group	Nuclei	Myelin	Synaptosomes	Mitochondria	Microsomes	Supernatant
0.25	$ACTH_{4-10}$	111.3±13.6(3)	97.1±16.8(3)	81.6±19.6(3)	74.2±32.2(3)	96.9±18.7(3)	
	CONTROL	90.7±15.1(3)	102.9±29.9(3)	119.6±29.6(3)	125.7±30.4(3)	103.1±18.9(3)	
1	$ACTH_{4-10}$	89.7±34.4(6)	99.1±21.9(6)	78.7±26.7(6)	84.9±20.4(6)	92.8±24.7(6)	98.5± 6.0(4)
	CONTROL	112.8±21.8(6)	100.9±42.8(6)	121.4±62.4(6)	115.2±47.9(6)	107.2±21.6(6)	101.5± 4.9(4)
2	$ACTH_{4-10}$	112.3±24.8(3)	107.7±11.6(3)	82.6±24.9(3)	100.7± 8.6(3)	73.8±15.2(3)	103.4± 5.4(3)
	CONTROL	87.7±24.5(3)	93.1± 7.4(3)	117.4±16.7(3)	99.3± 8.6(3)	126.2±15.3(3)	96.6± 9.3(3)
24	$ACTH_{4-10}$	80.8±13.2(4)	72.5±23.1(4)	118.5±20.9(4)	98.2± 4.1(4)	116.4±23.2(4)	77.3±20.1(4)
	CONTROL	119.1±24.6(4)	127.5±43.4(4)	81.5±32.2(4)	101.7± 5.1(4)	83.6±23.2(4)	122.7±18.7(4)
48	$ACTH_{4-10}$	97.1±18.9(4)	94.2±18.2(4)	99.3±20.1(4)	110.1± 8.9(4)	118.4±16.0(4)	104.7± 9.5(4)
	CONTROL	103.0±17.0(4)	105.7±20.1(4)	100.7±22.8(4)	89.9±15.3(4)	81.6±15.0(4)	96.4±12.3(4)
120	$ACTH_{4-10}$	88.3±22.8(4)	98.4±10.7(4)	89.6±23.2(4)	95.1±15.9(4)	101.1± 8.2(4)	85.0±19.1(4)
	CONTROL	111.7±17.7(4)	101.7±13.1(4)	110.5±21.6(4)	104.9±15.1(4)	98.9± 8.2(4)	115.0±15.2(4)
144	$ACTH_{4-10}$	101.7±11.1(4)	101.2±20.9(4)	100.4±14.1(4)	94.7± 5.6(4)	102.4±16.8(4)	80.3±18.6(4)
	CONTROL	98.3±18.5(4)	98.8±18.6(4)	99.5±12.4(4)	105.3± 7.3(4)	97.8± 9.7(4)	119.7±18.4(4)
168	$ACTH_{4-10}$	100.5±17.8(4)	105.9±15.5(4)	100.8± 4.9(4)	97.5±17.9(4)	94.1±15.0(4)	
	CONTROL	99.5±14.4(4)	94.2±17.3(4)	99.2± 3.2(4)	101.7±14.9(4)	105.9±16.8(4)	

Results expressed as mean % specific activity ± standard deviation (n)

The students t-test with Bessel's correction was used for the statistical analysis of the results.

that $ACTH_{4-10}$ stimulates incorporation of leucine into protein for use throughout the cell. These results give no information as to whether neuronal or glial cells are involved.

The microsomal fraction shows a rather lower specific activity of protein than that reported by other workers (e.g. Burdman and Journey, 1969) implying a high degree contamination with a fraction with a slow turnover of protein. A breakdown of the results indicated that although the absolute amount of label in the microsomal fraction was high the amount of protein present was also very high. The studies on the purity of the subcellular fractions described in Section 3(iii) indicated that the microsomal fraction was contaminated with the soluble supernatant fraction. This is consistant with the protein results and might account for the variable results obtained in the microsomal fraction.

SECTION 4

THE EFFECT OF $ACTH_{4-10}$ ON THE LEVELS OF FREE AMINO ACIDS IN RAT PLASMA AND BRAIN STEM

Introduction

Treatment of rats with $ACTH_{4-10}$ results in an increase in the incorporation of ^{14}C-leucine into protein of non cortical brain areas (Section 2). This increase could be due to an increase in availability of free amino acids, a decrease in protein breakdown, an increase in the synthesis of mRNA, an increase in RNA polymerase activity or an increase in the synthesis of proteins at the translational level. The first possibility was investigated in the experiments described below.

Many procedures have been shown to alter free amino acid levels, and thus alter protein synthesis, in peripheral tissues. For example, hydrazine inhibits amino acid degredation by interfering with transamination. Thus treatment of animals with hydrazine increases plasma and tissue levels of amino acids and liver protein synthesis is increased (Munro, 1970). Insulin, a peptide hormone, has been shown to decrease the plasma levels of all amino acids while increasing the levels of some amino acids in muscle (Wool, 1964). There is some evidence to show that the stimulatory effect of insulin on muscle protein synthesis is not caused by the increased free amino acid levels in the tissue (Stirewalt and Wool, 1966) although this has been disputed (Goldstein and Reddy, 1968).

It was possible that $ACTH_{4-10}$ increased protein synthesis by increasing the levels of free amino acids. The level of free amino acids in rat plasma and brain-stem was studied.

Method

The method for the estimation of free amino acids was based on that of Stein and Moore (1950). Amino acids were separated by ion exchange chromatography and estimated by their colour reaction with ninhydrin.

(i) Preparation of samples

Male, albino Wistar rats weighing 140-160 gm were randomly assigned to one of two groups.

$ACTH_{4-10}$ group received daily intraperitoneal injections of 20 µg $ACTH_{4-10}$ in 0.2 ml saline

Control group received daily intraperitoneal injections of 0.2 ml physiological saline.

On the 14th day of the experiment all rats were killed by decapitation. Blood was collected in tubes containing heparin to give a final concentration of approx. 20 iu/ml blood. The brain stem was rapidly dissected out and homogenized in acid as described below.

It is necessary to remove the protein from the samples as this clogs the resin and results in poor resolution of the amino acids. Numerous deproteinization techniques have been used. For example, Prescott and Waelsh (1941) used ultrafiltration, Stein and Moore (1954) picric acid,

Hamilton (1962) 3% sulphosalicylic acid and Gerok (1962) 10% trichloroacetic acid. Several authors have compared two or more methods drawing varying conclusions as to which method is best. Stein and Moore (1954) compared equilibrium dialysis, ultrafiltration and precipitation with picric acid for removal of protein from plasma. They decided that the picric acid method was best as it was the most reproducible and required the least time. Block et al., (1966) compared deprotinization of plasma by picric acid and sulphosalicyclic acid. They concluded that both methods gave similar results for amino acid levels and were both reproducible but that the sulphosalicylic acid method was the most convenient as it was necessary to extract the picric acid before analysis. It seems generally accepted that acid precipitation gives more reproducible results than the other methods used. All the acids studied seem to give similar results; the most appropriate acid being determined by the ease of its extraction from the amino acid solution. Precipitation of proteins with trichloroacetic acid (TCA) is widely used for brain tissue (e.g. Shimizu et al., 1966) and plasma (e.g. Gerok, 1962). TCA may easily be removed by extraction with 3:1 diethyl ether. This method was chosen for protein precipitation.

(a) Plasma

As soon as possible after the collection of the blood, it was centrifuged in a Mistral 2L (MSE) centrifuge to obtain the plasma supernatant. Plasma rather than serum was used because de Wolfe et al.(1967)

found that amino acids are more stable in heparinized plasma than serum. The volume of the plasma was measured and 15% TCA containing 1.2uM Nor leucine was added in the proportion of 1 volume TCA to 2 volumes plasma. Nor leucine was used as the internal standard as described below. The solution was mixed thoroughly and allowed to stand on ice for 10 min to permit complete precipitation of the proteins. The suspension was then centrifuged and the protein-free plasma removed. TCA was removed by extracting 3 times with 3 volumes of diethyl ether (Shimizu et al., 1966; Lowden and Laramée, 1969). The aqueous phase was stored at -20°C pending analysis.

(b) Brain stem

The brain stem samples were weighed and homogenized, in 4 ml 5% TCA containing 0.4 µM nor leucine, immediately after dissection. The mixture was allowed to stand in ice for 10 min and was then centrifuged. The protein-free supernatant was removed, the TCA extracted with diethyl ether, and the aqueous layer stored at -20°C until analysis.

(ii) Method of analysis

A Technicon automatic analyser, Model NC-1 (1966) was used for the separation and analysis of the amino acids.

The separation of amino acids was achieved on a column (0.4 x 4 ft.6 in) of Technicon Chromobeads B. The amino acids were eluted using a continuous gradient of citrate

buffers from pH 2.75 to pH 6.0. The method of separation was based on that of Piez and Morris (1960) and on the system recommended by Technicon. The composition of the buffers used is given in table 1.

TABLE 1 : Composition of elution buffers

	Buffer pH			
	2.75	3.0	3.8	6.0
Trisodium atrate (gm)	73.55	73.55	73.55	73.55
Sodium hydroxide (gm)	11.0	11.0	11.0	11.0
Ion-free water (ml)	4,500	4,800	4,800	4,800
Methyl cellusolve (ml)	350	-	-	-
Thiodiglycol (ml)	25	25	25	-
25% Brij 35 (ml)	50	50	50	50
Sodium chloride (gm)	-	-	-	292.5
Chloroform (drops)	2-3	2-3	2-3	2-3

All buffers were titrated with 6N HCl to give the appropriate pH and the volume was then made up to 5 litres.

The buffers were continuously dispensed from a Technicon 'autograd'. This vessel is based on the varigrad developed by Peterson and Sober (1959). It has ten connected compartments which were each filled with 80 ml buffer. Compartments 1 and 2 contained pH 2.75 buffer, 3 and 4 pH 3.0 buffer, 5 pH 3.8 buffer and compartments 6-10 pH 6.0 buffer. The column was kept at 60°C throughout. Methyl cellosolve was added to the pH 2.75 buffer as the addition of organic solvents has been reported to increase the resolution between serine and threonine (Thomson and Miles, 1964) and sodium chloride was added to the pH 6.0 buffer as

Kominz (1962) reported that increasing the sodium ion concentration increased the resolution of the basic amino acids.

Amino acids were detected in the eluate by the ninhydrin reaction. Even though ninhydrin is rather unstable in solution, it is widely used as the detecting reagent. Trinitrobenzene-sulphonic acid is an alternative. It is stable to both light and oxidation but gives no colour with proline or hydroxyproline. Originally, Spackman, Stein and Moore (1954) used methyl cellosolve as solvent and stannous chloride as reducing agent. Other reducing agents which have been used to form the necessary hydrindantin in solution are potassium cyanide and hydrazine sulphate. The system chosen here was that recommended by Technicon.

The eluate was automatically mixed with ninhydrin and hydrazine reagents using a Technicon proportionating pump. The composition of these reagents is given in table 2.

TABLE 2 : Composition of hydrazine and ninhydrin reagents.

Ninhydrin	Hydrazine
100 gm Ninhydrin	1.31 gm hydrazine sulphate
5,000 ml methyl cellosdue	2,500 ml methyl cellosolve
2,500 ml 4N Na Acetate buffer, pH 5.51	2,500 ml ion-free water
2,500 ml ion-free water	5 drops conc. H_2SO_4

The proportions of these reagents was approximately 3.5 volumes eluated: 4.5 volume ninhydrin: 3.0 volume hydrazine. Nitrogen bubbles were also added to the system.

The mixture was passed through a heating coil at 98°C for 20 min and was then cooled to room temperature in a further coil. The purple colour was then measured spectrophotometrically using a Technicon colourimeter which was arranged to give a trace on an Elliot linear recorder. A diagramatic representation of the system is given in fig. 1.

This system took approximately 16 hr to estimate all the amino acids in a sample of brain or plasma. After arginine, the last amino acid measured, had come off the column, the column was washed with 0.2N NaOH with E.D.T.A. for 20 min. Regenerating buffer (pH 2.75) was then pumped through the column for 1½hr.

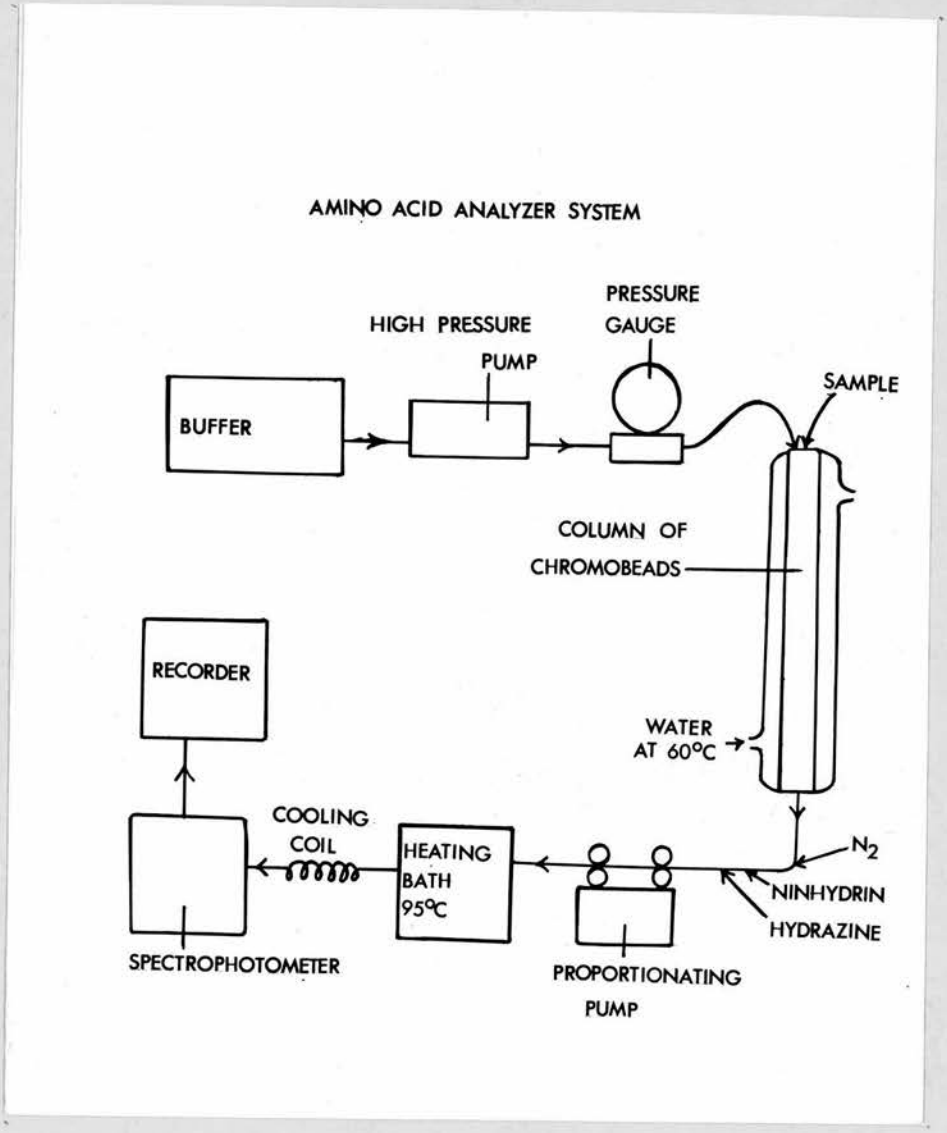
In total this system took approximately 20 hr. to analyse one sample and was sensitive in the range of 2-6 uM/ml for most amino acids. Using other systems, it is now possible to analyse protein hydrolysates in less than 4½ hr (Miller and Piez, 1966) with a detection limit of 10^{-10} mole (Hamilton, 1963). Although the equipment used here was neither very sensitive nor very fast it gave reproducible results and was sufficiently sensitive to measure a wide range of amino acids in plasma and brain stem.

(iii) Identification and quantification of amino acids

(a) Internal standard

The internal standard used was nor leucine as it is not found in either plasma or brain and does not interfere with the measurement of any other amino acids in our system. Walsh and Brown (1962) found nor leucine to be a reliable internal standard.

Fig. 1 : A diagramatic representation of the amino acid analyzer system.



(b) Identification of peaks

As the equipment used here had not been used for eight years, it was necessary to run rather more standard solutions of amino acids than would normally be required.

The approximate positions of the peaks could be estimated by reference to the literature (e.g. Dickinson et al., 1965) and the retention time was more accurately estimated by passing single or groups of amino acids, together with nor leucine, through the system.

(c) Sensitivity

This method of analysis was sensitive in the range 1-5 μM. The sensitivity varied with the amino acid, the system being most sensitive for glutamic acid (0.5 μM) and least for tryptophan. The retention time of tryptophan and the sensitivity varied from run to run and was therefore not suitable for estimation by this method. Other workers have also reported this (Knott et al., 1973).

After all the common amino acids had been identified, a standard mixture of 26 amino acids, each in a concentration of 4 uM was run through the machine, and the ratio of the area of the amino acid peak to the area of the peak produced by nor leucine was calculated. This was repeated several times. There was a slight variation in the sensitivity of the machine but it was found that the sensitivity changed to the same degree for all amino acids. That is, the ratio of the amino acid peak to the nor leucine peak was constant (table 3). Throughout this experiment the standard amino acid mixture was run at least once in every 8 experimental runs.

TABLE 3 : To show the reproducibility of estimation of standard amino acids

Amino Acid	Area of Nor leucine peak / Area of amino acid peak
Taurine	1.32 ± 0.10 (6)
Hydroxyproline	1.33 ± 0.06 (6)
Aspartic acid	1.05 ± 0.04 (6)
Threonine	1.09 ± 0.05 (6)
Serine	0.93 ± 0.06 (6)
Glutamic acid	0.68 ± 0.01 (6)
Proline	0.80 ± 0.01 (6)
Glycine	0.98 ± 0.005 (6)
Alanine	1.10 ± 0.02 (6)
Valine	1.18 ± 0.04 (6)
Cystine	0.41 ± 0.01 (6)
Methionine	0.98 ± 0.02 (6)
Isdeucine (i)	2.25 ± 0.05 (6)
(ii)	2.42 ± 0.06 (6)
Leucine	1.06 ± 0.04 (6)
Norleucine	1.00
Tyrosine	0.99 ± 0.01 (6)
Phenylalanine	0.92 ± 0.03 (6)
Hydroxylysine	0.63 ± 0.01 (6)
Lysine	0.48 ± 0.01 (6)
Histidine	0.79 ± 0.02 (6)
Arginine	1.02 ± 0.04 (6)

Results expressed as mean ± standard deviation (n)

The quantity of test amino acids was calculated by comparing the area of the trace for the test amino acid to the standard amino acids. Corrections were made for the varying sensitivity of the machine as seen by the change in area of the nor leucine peak.

Results

$ACTH_{4-10}$ treatment has no significant effect on the levels of free amino acids in rat plasma (table 4) or brain stem (table 5).

Discussion

The control values obtained were on the whole slightly lower but comparable with those of other workers (Mandel and Mark, 1965; Louden and La Ramie, 1969; Knott et al., 1972; Dickinson et al., 1965). There is a wide variation of values reported in the literature but as amino acid levels vary with the species of animal, sex, age, diurnal rhythms and other factors (Munro, 1970) this is hardly surprising.

$ACTH_{4-10}$ has no effect on free amino acid levels in the plasma and brain stem suggesting that it does not increase protein synthesis by increasing the availability of amino acids although the possibility that it increases the amino acid levels in one small brain area cannot be ruled out at present.

TABLE 4 : To show the effect of $ACTH_{4-10}$ on free amino acid levels in rat plasma.

Amino acid	Group	
	$ACTH_{4-10}$	Control
Taurine	1.31 ± 0.20 (6)	1.785 ± 0.43 (5)
Hydroxyproline	0.10 ± 0.01 (6)	0.211 ± 0.03 (5)
Aspartic acid	0.255 ± 0.09 (6)	0.24 ± 0.04 (5)
Threonine	0.615 ± 0.13 (6)	0.555 ± 0.11 (5)
Serine	0.495 ± 0.04 (6)	0.36 ± 0.06 (5)
Glutamic acid	0.675 ± 0.17 (6)	0.495 ± 0.14 (5)
Proline	0.795 ± 0.22 (6)	0.83 ± 0.23 (5)
Glycine	0.810 ± 0.12 (6)	0.64 ± 0.11 (5)
Alanine	0.84 ± 0.22 (6)	0.675 ± 0.14 (5)
Valine	0.165 ± 0.03 (6)	0.165 ± 0.01 (5)
Cystine	0.099 ± 0.035(6)	0.075 ± 0.03 (5)
Methionine	0.0995± 0.075(6)	0.090 ± 0.02 (5)
Isoleucine	0.225 ± 0.09 (6)	0.12 ± 0.08 (5)
Leucine	0.240 ± 0.02 (6)	0.32 ± 0.06 (5)
Tyrosine	0.15 ± 0.04 (6)	0.105 ± 0.02 (5)
Phenylalanine	0.105 ± 0.02 (6)	0.105 ± 0.03 (5)
Ornithine	0.186 ± 0.045(6)	0.120 ± 0.03 (5)
Lysine	0.632 ± 0.131 (6)	0.75 ± 0.08 (5)
Histidine	0.144 ± 0.018(6)	0.105 ± 0.03 (5)
Arginine	0.371 ± 0.097(6)	0.06 ± 0.09 (5)

Results expressed as mean µM/ml ± standard deviation (n)

TABLE 5 : To show the effect of $ACTH_{4-10}$ on free amino acid levels in rat brain stem

Amino Acid	Group	
	$ACTH_{4-10}$	Control
Taurine	1.238 ± 0.233	1.288 ± 0.244
Aspartic Acid	1.166 ± 0.155	1.012 ± 0.11
Threonine	0.352 ± 0.048	0.324 ± 0.025
Glutamic Acid	2.134 ± 0.222	2.14 ± 0.178
Glycine	0.446 ± 0.121	0.512 ± 0.012
Alanine	0.118 ± 0.017	0.142 ± 0.012
Valine	0.048 ± 0.01	0.064 ± 0.017
Lysine	0.254 ± 0.066	0.308 ± 0.28
Arginine	0.162 ± 0.046	0.174 ± 0.030

Results expressed as mean µM/gm wet weight of brain ± standard deviation

Each result is the mean of 6 results.

SECTION 5

THE EFFECT OF $ACTH_{4-10}$ AND $ACTH_{4-10}$-7-D-phe ON THE INCORPORATION OF ^{14}C-LEUCINE INTO RETINAL AND BRAIN STEM PROTEIN IN VITRO.

Introduction

$ACTH_{4-10}$ has been shown to increase brain protein synthesis in intact rats (Section 2) while having no effect on nucleic acid metabolism (Dewar, 1972). As $ACTH_{4-10}$ has no effect on the overall levels of amino acids in the brain (Section 4) it seems probable that it is acting either by increasing transport of amino acids across the cell membrane to the site of protein synthesis or is directly affecting protein synthesis at the translational level. *In vitro* experiments were designed to investigate whether ACTH analogues act by these mechanisms.

I. To show the effect of ACTH analogues on the incorporation of ^{14}C-leucine into retinal protein *in vitro*

In the first instance, the retina was chosen for the *in vitro* system. It has the same embryological origin as the brain (ectodermal) and may easily be dissected intact from the eye. Unlike slices of brain tissue, it suffers little damage in the dissection and consequently there is less leakage of material from the cells.

A. General method

Male, albino Wistar rats weighing 140-160 gm were used throughout.

(i) Dissection of the retina

The rat was killed by decapitation, the eyes removed as quickly as possible and placed in a petri dish containing ice cold, oxygenated, incubation medium. Each eye was then placed on a perspex block which had indentations of such a size that the eye fitted snuggly up to the level of the corneoscleral junction. The eye was held in position with another perspex block and was cut in half by quickly passing a long razor blade between the two blocks. The posterior half of the eye was gripped at the side with a pair of fine forceps and returned to the incubation medium where it was held retinal side down and the retina was teased out by stroking the back of the eye.

The whole procedure from the death of the rat to the finished dissection of both retinae took approximately two minutes.

(ii) Incubation procedure

Each retina was preincubated in 2 ml of incubation medium at 37°C for a predetermined time. The reaction was started by the addition of 1.25 uCi L-1-^{14}C leucine (60 mCi/mmol, Radiochemical Centre, Amersham) in 50 µl saline and stopped after a set incubation time by cooling the tube in methanol/solid CO_2 mixture. The tubes were immediately spun in a Mistral 2L centrifuge (MSE) to sediment the retina. 20 µl of the medium was reserved for estimation of its radioactivity by the method described in Section 1 (iv). The rest of the medium was discarded.

The retina was washed twice by agitation in ice cold saline for several minutes. It was sedimented between each wash by centrifuging in the Mistral 2L. The retina was homogenized in 1 ml 10% TCA. The homogenate was transferred to an Eppendorf tube and centrifuged on an Eppendorf microcentrifuge. The supernatant, the acid soluble fraction, was reserved for measuring the level of radioactivity, while carbohydrate and lipid-free protein and nucleic acids were extracted from the precipitate as described in section 1 (iii). The level of radioactivity in the medium, acid soluble fraction and protein was measured and the DNA and protein were estimated as described in Section 1 (iv) and in the Appendix.

All procedures up to the homogenization of the retinae were carried out in Cl4 test tubes (Quick-fit) unless otherwise stated in the experiment. As the retinae were to be separated from the medium by centrifugation, it was rather more convenient to use a vessel that could be centrifuged rather than the conical flasks used by most workers (e.g. Goodchild and Neal, 1973; Kiely and Sourkes, 1972). The tubes were maintained at 37°C using a 'Dri block' (Techne (Cambridge) Ltd.) heating block. The medium was gently gassed throughout with the appropriate gas applied through pasteur pipettes fixed to a 12 outlet manifold attached to the gas cylinder. The gassing agitated the medium making further shaking unnecessary.

(iii) The incubation medium

Two different incubation media have been used by various workers. Some favour Krebs original Ringer phosphate medium (e.g. Chain et al., 1962) while others favour Krebs-Henseleit original Ringer bicarbonate (e.g. Goodchild and Neal, 1973).

To decide which medium was most appropriate for the incorporation of ^{14}C-leucine into protein, an experiment was designed to compare the two media.

Method

Thirtythree retinae were divided into two groups. Thirteen were incubated in 'bicarbonate' medium and twenty were incubated in 'phosphate' medium. The composition of the media is given in table 1. 0.1% glucose was added to both media. The bicarbonate medium was gassed with 95% O_2/5% CO_2 while the phosphate medium was gassed with 100% O_2.

Approximately half the retinae in each group were preincubated for 10 min and half for 30 min. All were incubated for 30 min after the addition of ^{14}C-leucine.

Results

Table 2 shows the specific activity of protein in retinae preincubated for either 10 or 30 min in bicarbonate or phosphate medium. Specific activity may be defined as d.p.m. per mg acid insoluble protein extracted. The protein specific activity remains constant when retinae are preincubated for 10 or 30 min in bicarbonate medium but

TABLE 1 : To show the composition of Krebs-Henseleit original Ringer bicarbonate medium (known as bicarbonate medium) and Krebs original Ringer phosphate medium (known as phosphate medium)

	% w/v	Molarity	Parts by volume bicarbonate	Parts by volume phosphate
NaCl	0.9%	0.154M	100	100
KCl	1.15%	0.154M	4	4
$CaCl_3$	1.22%	0.11M	3	3
KH_2PO_4	2.11%	0.154M	1	-
$MgSO_4.7.H_2O$	3.82%	0.154M	1	1
$NaHCO_3$ *	1.3%	0.154M	21	-
Phosphate buffer**		0.1M		21

* Gassed with 100% CO_2 for 1 hr before making with the other solutions.

** 17.8 gm NA_2HPO_4 $2H_2O$ + 20 ml 1N HCl made up to 1 litre pH = 7.4.

TABLE 2 : To show the effect of the composition of the incubation medium on the incorporation of ^{14}C-leucine into retinal protein.

Length of preincubation time	Medium: Bicarbonate	Medium: Phosphate
10 min	74,650 ± 8,385(7)	41,675 ± 17,965(11)
30 min	67,495 ± 13,830(6)	26,540 ± 22,200(9)

Results expressed as mean specific activity ± standard deviation
Number of retinae in parenthesis.

it is markedly reduced after a 30 min preincubation in phosphate medium. At both preincubation times the incorporation of ^{14}C-leucine into protein of retinae incubated in phosphate medium is significantly lower than that in retinae incubated in bicarbonate medium (10 min, $p < 0.001$; 30 min, $p < 0.01$).

The level of radioactivity in the acid soluble fraction is slightly lower after 30 min preincubation in phosphate medium than 10 min, but this difference is not significant (table 3). The specific activity in the acid soluble fraction of the phosphate medium is rather higher than from the bicarbonate medium. Specific activity in the acid soluble fraction may be defined as d.p.m. in the acid soluble fraction/mg DNA present in the retina.

TABLE 3 : To show the differences in the level of radioactivity in the acid soluble fraction after incubating retinae in phosphate and bicarbonate medium.

Length of preincubation	Medium	
	Bicarbonate	Phosphate
10 min	269,318 ± 49,204(7)	389,713 ± 165,984(11)
30 min	275,755 ± 97,314(6)	303,462 ± 146,665(9)

Results as mean acid soluble specific activity ± standard deviation (n).

Discussion

The difference in specific activity of protein after varying preincubation times should give an indication of the damage caused by the incubation conditions. The specific activity remains constant when retinae are incubated in bicarbonate medium but is markedly reduced after 30 min. preincubation in phosphate medium, implying that the phosphate medium is less suitable for the incorporation of leucine into protein than the bicarbonate medium. Also at both times the incorporation of leucine into retinal protein is lower in retinae incubated in phosphate medium than in bicarbonate medium.

The low level of protein synthesis in retinae incubated in phosphate medium is not due to reduced uptake of leucine as, in fact, the level of label in the acid soluble fraction is higher in these retinae than those incubated in bicarbonate medium. This difference could be due to the same uptake of leucine but a reduced utilization of it when the retinae are incubated in unfavourable conditions. The differences in the acid soluble fraction are perhaps rather too large to be accounted for by this suggestion alone. The high levels of radioactivity in the acid soluble fraction could perhaps also be due to an increased membrane permeability due to tissue damage in the phosphate incubated retinae.

These results agree with those of Bassi and Bernelli-Zazzera (1960) who reported that incubation of brain slices in a potassium-rich phosphate medium increases respiration

but inhibits amino acid incorporation into protein.

Krebs-Henseleit original Ringer bicarbonate medium was chosen for all subsequent *in vitro* experiments.

(iv) To demonstrate that glucose is necessary for satisfactory incorporation of ^{14}C-leucine into brain protein in vitro

Glucose is normally added to the salt medium to maintain aerobic respiration (Reading and Sorsby, 1964; Goodchild and Neal, 1973).

Method

Thirty-two retinae were divided into two groups, 16 being incubated in glucose-free medium and 16 being incubated in medium containing 0.1% glucose. These groups were subdivided into 2 further groups - 8 retinae from each group being incubated for 30 min with ^{14}C-leucine and 8 for 60 min. All were preincubated for 10 min. The method for incubation of the retinae and extraction and estimation of the protein was as in Section 5 (ii).

Results

It may be seen from table 4 that retinae incubated in glucose-free medium incorporate markedly less leucine than those incubated in medium with added glucose.

TABLE 4 : To show the requirement for glucose in the incubation medium.

Medium	Incubation Time	
	30 min.	60 min.
Glucose-free	20,085 ± 13,085(8)	26,660 ± 15,760(8)
0.1% glucose	76,140 ± 14,030(8)	241,910 ± 38,455(8)

Results expressed as mean specific activity ± standard deviation.

Number of retinae in parenthesis.

Discussion

Addition of glucose to the salt medium is necessary for *in vitro* protein synthesis, and was added to the medium in a final concentration of 0.1% in all the experiments described below.

(v) Incubation Time

To show the time course of incorporation of ^{14}C-leucine into retinal protein *in vitro*.

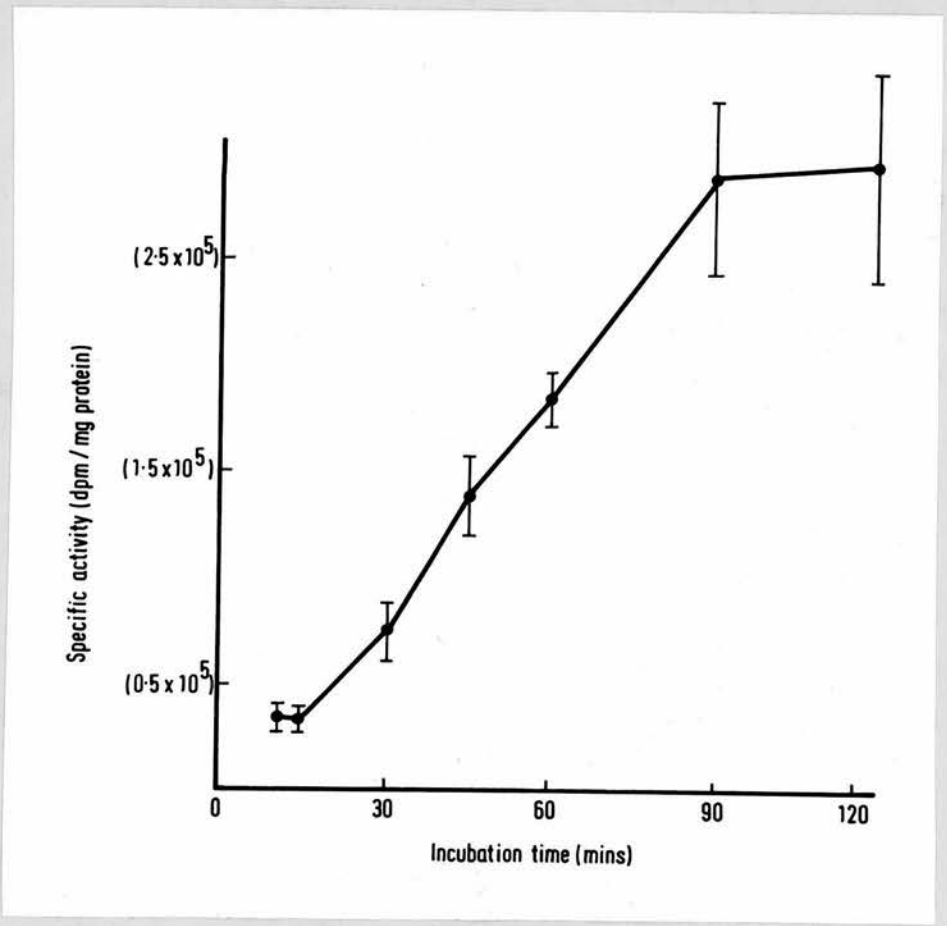
Method

Twenty-eight retinae from 14 albino Wistar rats were preincubated in bicarbonate medium for 10 min. The reaction was started by the addition of ^{14}C-leucine and was stopped by cooling after various predetermined incubation times. The protein was extracted and estimated as described in Section 5A(ii).

Results

Fig. 1 shows the dpm/mg extracted TCA insoluble protein after various incubation times. It may be seen

Fig. 1 : To show the time course of incorporation of ^{14}C-leucine into retinal protein in vitro.



Each point represents the mean of 4 results.

The bar lines indicate standard deviation.

that the incorporation of leucine into protein is linear between 15 and 90 min. incubation. Incorporation is reduced after 90 min of incubation.

Discussion

The in vitro incorporation of ^{14}C-leucine into retinal protein is linear with time over the range 15 to 90 min. After 90 min. the incorporation seems to level off, possibly due to the lack of some essential nutrient (e.g. ATP). 30 and 60 min. incubation times were chosen for all subsequent experiments.

B. To show the effect of $ACTH_{4-10}$ and $ACTH_{4-10}$-7-D-phe on the incorporation of ^{14}C-leucine into retinal protein in vitro.

(i) To show the effect of 0.1 and 1 ug/ml $ACTH_{4-10}$ on retinal protein synthesis in vitro.

Method

Retinae were divided into three groups:

Control groups were incubated in standard bicarbonate medium

$ACTH_{4-10}$ (0.1) group were incubated in bicarbonate medium with $ACTH_{4-10}$ to give a final concentration of 0.1 ug/ml $ACTH_{4-10}$.

$ACTH_{4-10}$ (1) group were incubated in bicarbonate medium with $ACTH_{4-10}$ to give a final concentration of 1 µg/ml $ACTH_{4-10}$.

In both of the latter two groups part of the NaCl was replaced by $ACTH_{4-10}$ dissolved in saline to give the appropriate concentration.

All retinae were preincubated in the appropriate medium for 10 min. and after the addition of labelled leucine some from each group were incubated for 30 min. and some for 60 min. Various fractions were extracted and estimated as described in section 5A(ii).

Results

It may be seen that $ACTH_{4-10}$, in the concentrations used here, has no effect on the incorporation of ^{14}C-leucine into retinal protein in vitro (table 5) or on the level of radioactivity in the acid soluble fraction (table 6).

TABLE 5 : To show the effect of 0.1 and 1.0 µg/ml $ACTH_{4-10}$ on retinal protein synthesis in vitro.

Group	Incubation Time 30 min.	60 min.
Control	76,980 ± 30,370(17)	241,910 ± 38,455(10)
$ACTH_{4-10}$(0.1)	73,475 ± 27,365(17)	211,895 ± 39,290(10)
$ACTH_{4-10}$(1.0)	77,885 ± 31,900(6)	209,920 ± 33,145(6)

Results expressed as mean dpm/mg protein ± standard deviation (number of retinae).

TABLE 6 : To show the effect of $ACTH_{4-10}$ on the level of label in the acid soluble fraction of retinae incubated in vitro.

Group	Incubation Time 30 min.	60 min.
Control	287,957 ± 100,739(17)	276,452 ± 74,371(10)
$ACTH_{4-10}$(0.1)	318,167 ± 142,248(17)	248,717 ± 49,428(10)
$ACTH_{4-10}$(1.0)	345,328 ± 63,837(6)	259,764 ± 52,322(6)

Results expressed as mean dpm/mg DNA ± standard deviation (n).

Discussion

$ACTH_{4-10}$ in concentrations of 0.1 or 1 µg/ml has no effect on retinal protein synthesis in vitro.

(ii) To show the effect of varying the length of pre-incubation of the retina with $ACTH_{4-10}$ on the effect of $ACTH_{4-10}$ on in vitro protein synthesis in the retina.

In the previous experiment (section 51 B (i)) the retinae were preincubated in medium containing $ACTH_{4-10}$ for 10 min prior to the addition of ^{14}C-leucine. 10 min. was chosen as it was long enough to allow the retinae and medium to reach 37°C whilst subjecting the retinae to the minimum length of time in the medium thus reducing tissue damage. This length of time might not be sufficient to allow the peptide or possibly its messenger to enter the retina or to act once they had reached their site of action. An experiment was designed to test this hypothesis.

Method

Retinae were divided into 2 groups, half being incubated in standard bicarbonate medium and half in medium containing 0.1 ug/ml $ACTH_{4-10}$. The retinae were preincubated in the appropriate medium at 37°C for 10, 20 or 30 min. and the reaction started by the addition of ^{14}C-leucine. All retinae were then incubated for 30 min, the retinal protein being extracted and estimated as before (section 5 A (ii)).

Results

Table 7 shows that, even after 30 min preincubation with the retinae, $ACTH_{4-10}$ does not increase the incorporation of ^{14}C-leucine into retinal protein.

TABLE 7 : To show the effect of preincubating the retina with $ACTH_{4-10}$ for varying times, on the effect of $ACTH_{4-10}$ on retinal protein synthesis.

Preincubation time(Minutes)	Group	Specific Activity of Protein dpm/mg protein
10	$ACTH_{4-10}$	65,855 ± 18,840(7)
	Control	74,650 ± 14,455(7)
20	$ACTH_{4-10}$	78,155 ± 11,910(4)
	Control	69,075 ± 13,130(4)
30	$ACTH_{4-10}$	75,045 ± 25,700(7)
	Control	67,495 ± 31,580(7)

Results expressed as mean specific activity ± standard deviation (number of retinae).

Discussion

Even after preincubating the retina with $ACTH_{4-10}$ for 30 min, $ACTH_{4-10}$ has no effect on retinal protein synthesis.

(iii) To show the effect of 0.1 µg/ml $ACTH_{4-10}$-7-D-phe on retinal protein synthesis in vitro

Introduction

$ACTH_{4-10}$-7-D-phe has been shown to have the opposite behavioural effects to $ACTH_{4-10}$ in that it facilitates the extinction of a conditioned avoidance response in intact rats (Bohus and de Wied, 1966). As $ACTH_{1-10}$-7-D-phe causes a decrease in protein synthesis in the brain stem of hypophysectomized rats whereas $ACTH_{1-10}$ causes an increase (Schotman et al., 1972), one might expect that

$ACTH_{4-10}$-7-D-phe will cause a decrease in protein synthesis in the intact rat. $ACTH_{4-10}$-7-D-phe did tend to decrease the incorporation of ^{14}C-leucine into brain protein *in vivo* (Section 2 (vi)).

The effect of $ACTH_{4-10}$-7-D-phe on retinal protein synthesis was investigated.

Method

Sixteen retinae were randomly assigned to one of two groups.

$ACTH_{4-10}$-7-D-phe group were incubated in bicarbonate medium containing 0.1 ug/ml $ACTH_{4-10}$-7-D-phe.

Control group were incubated in standard bicarbonate medium.

Four retinae from each group were incubated for 30 min. and 4 for 60 min. All were preincubated for 10 min. The incubation procedure and the extraction and estimation of protein and DNA was as described in Section 5 1 A (ii).

Results

It may be seen from table 8 that 0.1 µg/ml $ACTH_{4-10}$-7-D-phe has no effect on the incorporation of ^{14}C-leucine into retinal protein *in vitro*.

Discussion

$ACTH_{4-10}$-7-D-phe, like $ACTH_{4-10}$, has no effect on the retinal protein synthesis *in vitro* when present in a concentration of 0.1 µg/ml.

TABLE 8 : To show the effect of 0.1 µg/ml $ACTH_{4-10}$-7-D-phe on retinal protein synthesis in vitro.

Group	Incubation Time 30 min.	60 min.
$ACTH_{4-10}$-7-D-phe	63,620 ± 17,640(4)	241,380 ± 31,010(4)
Control	66,895 ± 20,130(4)	272,150 ± 21,540(4)

Results expressed as mean specific activity ± standard deviation (number of retinae).

(iv) To show the effect of a series of daily injections of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into protein in vitro

As it is necessary to treat rats with a series of daily injections to produce any behavioural changes (Organon, Personal communication) or changes in protein metabolism in vivo (Section 1 (ii) a), it seemed possible that this number of injections was necessary to produce any change in protein metabolism in vitro. Rats were therefore treated with $ACTH_{4-10}$ for 2 weeks prior to the removal and incubation of their retinae.

Method

Fourteen, male, albino Wistar rats were randomly assigned to one of 2 groups.

$ACTH_{4-10}$ group received daily intraperitoneal injections of 0.2 ml of a neutral solution containing 20 µg $ACTH_{4-10}$ in physiological saline.

Control group received daily intraperitoneal injections of 0.2 ml of physiological saline.

On the 14th day of the experiment the rats were killed and their retinae removed. All the retinae were then pre-incubated in standard bicarbonate medium for 10 min. and were then incubated for either 30 or 60 min. $ACTH_{4-10}$ was not present in the medium. Protein was extracted from the retinae and estimated as described before (Section 5 I A (ii)).

Results

A series of injections of $ACTH_{4-10}$ has no effect on the incorporation of ^{14}C-leucine into retinal protein *in vitro* (table 9) or on the free pool of amino acids as estimated by the acid soluble fraction (table 10).

TABLE 9 : To show the effect of pretreatment of rats with $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into retinal protein in vitro

Incubation Time (Minutes)	Group	Specific Activity of Protein dpm/mg protein
30	$ACTH_{4-10}$	73,025 ± 33,760 (9)
	Control	81,530 ± 24,720 (9)
60	$ACTH_{4-10}$	185,985 ± 60,830 (4)
	Control	170,750 ± 44,435 (4)

Results expressed as mean dpm/mg protein ± standard deviation (Number of retinae)

TABLE 10 : To show the effect of pretreatment of rats with $ACTH_{4-10}$ on the level of label in the acid soluble fraction of retinae incubated with ^{14}C-leucine.

Incubation Time (Minutes)	Group	Acid soluble fraction d.p.m./mg DNA
30	$ACTH_{4-10}$	295,213 ± 47,984 (9)
	Control	201,424 ± 79,072 (9)
60	$ACTH_{4-10}$	283,637 ± 95,496 (4)
	Control	295,589 ± 72,235 (4)

Results expressed as mean dpm/mg DNA ± standard deviation (Number of retinae).

Discussion

It seems that pretreating rats with $ACTH_{4-10}$ has no effect on retinal protein. synthesis in vitro.

C. General Discussion

ACTH peptides in concentrations of 0.1 or 1.0 µg/ml have no effect on retinal protein synthesis even when the retinae are preincubated in drug solution for 30 minutes. This suggests that $ACTH_{4-10}$ acts by a mechanism other than on the protein synthetic machinery or on local transport of amino acids, that the action of $ACTH_{4-10}$ is so slow it cannot be detected by this method or that under these conditions the retina is relatively insensitive to the action of $ACTH_{4-10}$ possibly requiring extremely high doses

to produce a response. Another possibility is that the retina, although it has the same embryological origin as the brain, does not respond to $ACTH_{4-10}$ in the same way as the brain. This seems probable as it was shown in Section 2 that different brain areas respond differently to peptide treatment.

Pretreatment of rats with $ACTH_{4-10}$ did not affect retinal protein synthesis, suggesting that long treatment does not change amino acid levels or the protein synthetic machinery in the retina.

II. To investigate the effect of ACTH peptides on the incorporation of ^{14}C-leucine into brain stem protein in vitro.

Introduction

MSH has been shown to delay the extinction of a conditioned avoidance response in intact rats but in rats with bilateral lesions in the thalamic parafascicular area it fails to do so (Bohus and de Wied, 1967b). Lesions in other brain areas have no effect. When $ACTH_{1-10}$ is implanted in the rostral mesencephalon or the caudal diencephalon at the posterior thalamic level it delays the extinction of a conditioned avoidance response in a manner similar to that of systemic administration, but when it is implanted into other sites it is ineffective (Van Wimersma Greidanus and de Wied, 1969, 1971). These results indicate that certain brain stem areas are primarily involved in the behavioural response to MSH and other similar peptides. This is also the brain area where $ACTH_{4-10}$ increased the incorporation of leucine into protein *in vivo* (Section 2).

ACTH peptides had no effect on protein synthesis in the retina *in vitro* (Section 5 I). It is possible that the retina is rather less sensitive to the actions of ACTH peptides than is the brain itself. Experiments were therefore designed to investigate the effect of $ACTH_{4-10}$ and $ACTH_{4-10}$-7-D-phe on protein metabolism in rat brain stem slices.

A. General Method

Male, albino Wistar rats weighing 140-160 gm were used throughout.

(i) Preparation of slices

The rat was killed by decapitation and the brain removed as rapidly as possible. The brain stem was dissected from the rest of the brain by the method of Gispen et al., 1972 and was then cut into slices 0.3 mm thick using a McIlwain chopper (McIlwain and Buddle, 1953). The sliced brain was transferred to a petri dish containing oxygenated medium where the slices were separated using two fine dissecting needles. Slices were selected from the central region of the brain stem. These were from the diencephalon and included thalamic and hypothalamic nuclei with part of the caudate nucleus. The outer limits of the area taken are shown in fig. 2. These slices were transferred individually to test tubes containing 2 ml medium.

(ii) Incubation procedure

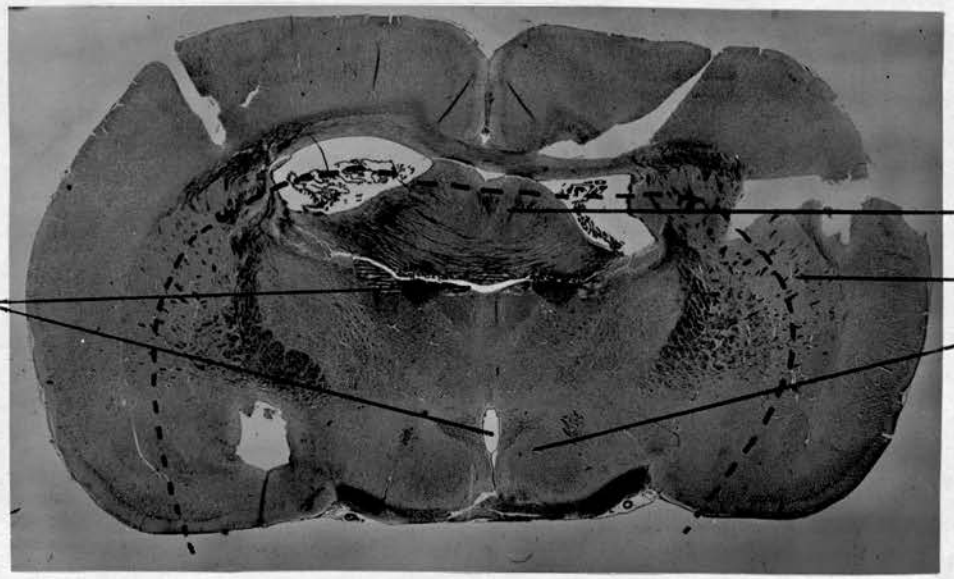
The method employed for incubating the slices and extraction and estimation of protein and DNA was the same as for the retina (Section 5 I A (ii)).

Slices were preincubated at 37°C for 10 min (unless stated otherwise in the experiment) and the reaction was started by the addition of 1.25 µCi ^{14}C-leucine in 50 µl saline. The slices were then incubated for a predetermined time, the reaction being stopped by cooling.

Krebs-Henseleit original Ringer bicarbonate medium (Dawson et al., 1959) (Bicarbonate medium) was chosen as the incubation medium as Krebs original Ringer phosphate medium has been reported to reduce amino acid incorporation

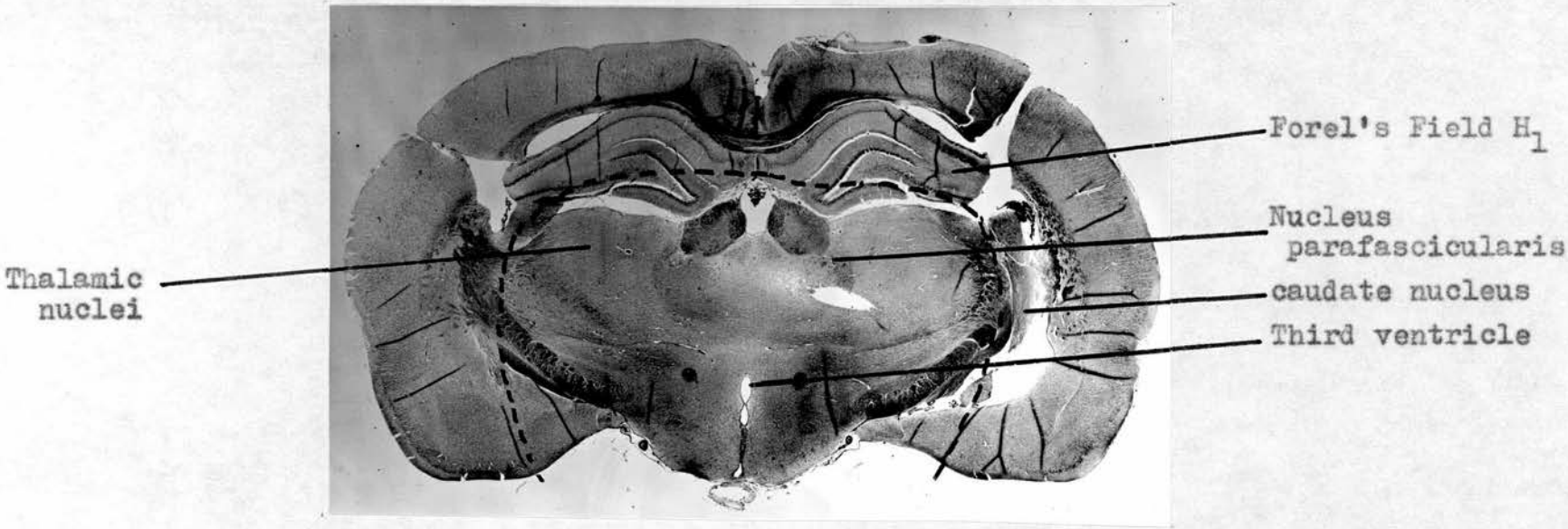
Fig. 2 : Transverse section of whole brain to show the limits of the brain area selected for the preparation of brain stem slices.

a) Rostral limit



--- Indicates the cutting line for removal of cortex.

Fig. 2. b) Caudal limit



---- Indicates the cutting line for removal of the cortex. This was estimated by comparison with slices in which the 'cortical area' had been removed.

Both slices (fig. 2a and b) were stained with Luxol fast blue and counterstained with H. and E.

in brain slices (Bassi and Bernelli-Zazzera, 1960). 0.1% glucose was present in the incubation medium in all experiments.

After incubation the slices were separated from the medium and were then washed twice with ice cold medium to remove any ^{14}C-containing medium adhering to the outside of the slice. As there is a substantial amount of leakage of free amino acids during this washing (Dunlop et al., 1974) the level of radioactivity in the washes was measured. This was used, together with the level of label in the acid soluble fraction, to give an indication of the uptake of leucine into the slices. In all experiments both control and treated slices were present and great care was taken to ensure that the washing procedure was the same for all slices.

(iii) Incubation time

(a) To show the time course of incorporation of ^{14}C-leucine into rat brain stem protein in vitro.

Method

Brain stem slices were prepared as above. All were preincubated in bicarbonate medium for 10 min. The reaction was started by the addition of ^{14}C-leucine and was stopped by cooling after various predetermined incubation. The protein was extracted and estimated as described in Section 5 I A (ii).

Results

The incorporation of ^{14}C-leucine in the brain stem

protein increases with time (fig.3). The rate of incorporation is greatest in the first 60 min levelling off after this.

Discussion

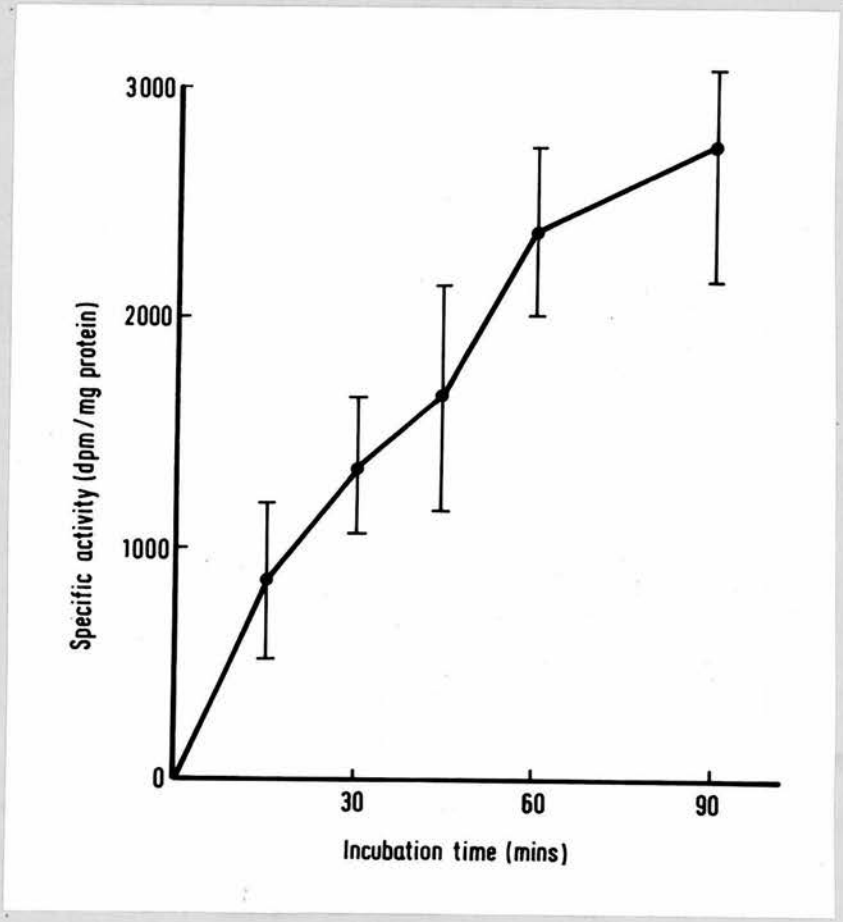
It seems that brain stem slices are capable of synthesizing protein under the conditions described here. The incorporation of leucine into protein is approximately linear up to a 60 min. incubation period but, between 60 and 90 min, the rate of incorporation levels off.

If the incorporation of leucine into brain stem protein is compared with the incorporation into retinal protein (Section 5 I A (v) fig. 1) it may be seen that the levels of radioactivity are considerably lower in brain stem protein after all incubation times. Low rates of incorporation of amino acids into brain tissue slices has been reported by other workers (Jones, 1972) presumably due to the nature of the tissue and due to tissue damage caused by the slicing procedure. In the experiments described below, the difference between treated and control samples was required rather than the absolute values of specific activity. It was felt that this method of incubating slices was adequate for this purpose.

(b) To show the effect of varying the preincubation time on the incorporation of ^{14}C-leucine into brain stem protein in vitro.

The effect of varying the preincubation time on the incorporation of leucine into protein during a fixed incubation time should give an indication of the degree of

Fig. 3 : To show the time course of incorporation of ^{14}C-leucine into rat brain stem protein, in vitro.



damage caused by the incubation conditions. As there is a low rate of incorporation, it was of interest to discover if this was due to the incubation procedure.

Method

Twelve brain stem slices were assigned to 1 of 3 groups. All were preincubated in standard bicarbonate medium but the length of this preincubation period varied. A third were preincubated for 10 min, a third for 20 min and a third for 30 min. After the addition of 1.25 uCi ^{14}C-leucine, all were incubated for 30 min. The reaction was stopped by cooling and the protein extracted and estimated as described before (Section 5 II A (ii)).

Results

It may be seen from table 11 that there is a tendency for the level of incorporation of ^{14}C-leucine into protein to decrease with increasing preincubation but there is no statistically significant difference between the groups.

TABLE 11 : To show the effect of varying the preincubation time on the incorporation of ^{14}C-leucine into protein of brain stem slices in vitro.

Preincubation Time (Minutes)	Specific Activity (dpm/mg protein)
10	2342.0 ± 823.5(4)
20	2029.5 ± 474.0(4)
30	1943.5 ± 372.5(4)

The results are expressed as mean specific activity ± standard deviation (number of slices).

Discussion

Varying the length of the preincubation period of slices in medium has no significant difference on the incorporation of leucine into slice protein, suggesting that these incubation conditions are suitable studying brain protein synthesis. There is a slight tendency for the incorporation to decrease with increasing preincubation time so for the experiments described below the incubation and preincubation times were kept as short as possible.

B. Experiments to show the effect of $ACTH_{4-10}$ and $ACTH_{4-10}$-7-D-phe on the incorporation of ^{14}C-leucine into brain stem protein in vitro.

(i) To show the effect of 0.5 and 1 ug/ml $ACTH_{4-10}$ on brain stem protein synthesis in vitro

Method

Brain stem slices were divided into three groups:

Control group: slices were incubated in standard bicarbonate medium

$ACTH_{4-10}$ (0.5) group: slices were incubated in bicarbonate medium with $ACTH_{4-10}$ to give a final concentration of 0.5 µg/ml $ACTH_{4-10}$

$ACTH_{4-10}$ (1) group: slices were incubated in bicarbonate medium with $ACTH_{4-10}$ to give a final concentration of 1.0 µg/ml $ACTH_{4-10}$.

In both the latter 2 groups part of the NaCl solution in the medium was replaced by $ACTH_{4-10}$ dissolved in saline to give the appropriate concentration of peptide. All slices were preincubated for 10 min but some samples were incubated for 30 min and some for 45 min after the addition of 1.25 µCi ^{14}C-leucine. The reactions were stopped by cooling and protein extracted and estimated as in Section 5 I A (ii). It should be noted that few slices may be obtained from each rat. It was therefore necessary to use a group of rats for this experiment. Slices to be treated and used as controls were randomly taken from each rat. Because of the limitations of the equipment it was also necessary to carry out the experiment in a series of batches. Controls were present in each batch.

Results

After both 30 and 40 min incubations there is a greater degree of incorporation of ^{14}C-leucine into slices treated with $ACTH_{4-10}$ than control slices. The difference between $ACTH_{4-10}$ treated and control slices is satistically significant (table 12). There is no difference in the uptake of ^{14}C-leucine by peptide treated and control slices (table 13).

TABLE 12 : To show the effect of $ACTH_{4-10}$ on the incorporation of ^{14}C-leucine into brain stem protein in vitro

Group	Incubation Time 30 min.	45 min.
Control	1092.5 ± 598.5(36)	1760.0 ± 493.0(16)
$ACTH_{4-10}$ (0.5)	1801.5 ± 333.5(12)*	2370.5 ± 616.5(12)**
$ACTH_{4-10}$ (1.0)	2003.5 ± 551.5(12)*	

The results are expressed as mean dpm/mg protein ± standard deviation (The number of slices)
* $p < 0.001$ ** $p < 0.01$

TABLE 13 : To show the effect of $ACTH_{4-10}$ on the amount of ^{14}C-leucine taken up by brain stem slice during an incubation

Group	Incubation Time 30 min.	45 min.
Control	$3.214 \times 10^6 \pm 1.11 \times 10^6$(36)	$2.675 \times 10^6 \pm 0.751 \times 10^6$(16)
$ACTH_{4-10}$(0.5)	$2.896 \times 10^6 \pm 0.764 \times 10^6$(12)	$2.956 \times 10^6 \pm 0.449 \times 10^6$(12)
$ACTH_{4-10}$(1.0)	$3.182 \times 10^6 \pm 0.942 \times 10^6$(12)	

The results are expressed as mean dpm/mg DNA ± standard deviation (number of slices)

Discussion

$ACTH_{4-10}$ increases the synthesis of brain stem protein in vitro. This effect is not due to an increase in the availability of labelled leucine, as there was no change in its uptake into the slice.

These results suggest that $ACTH_{4-10}$ is capable of acting directly in the brain rather than by an intermediate in some other part of the body. As the brain stem but not the retina is sensitive to $ACTH_{4-10}$ at a dose of 1 µg/ml it seems that either the brain stem is more sensitive to the action of $ACTH_{4-10}$ or that the peptide effect is specific to only certain areas of the central nervous system. This would agree with the behavioural studies reported above.

(ii) To show the effect of 0.5 and 1.0 ug/ml $ACTH_{4-10}$-7-D-phe on brain stem protein synthesis in vitro

In vivo, $ACTH_{1-10}$-7-D-phe decreases the incorporation of ^{14}C-leucine into brain stem protein in the hypophysectomized rat (Schotman et al., 1972) and $ACTH_{4-10}$-7-D-phe tends to decrease incorporation of leucine into protein of the same brain area in the intact rat (Section 2 (vi)). It was therefore of interest to investigate the effect of $ACTH_{4-10}$-7-D-phe on brain stem protein synthesis in vitro.

Method

Brain stem slices were randomly assigned to one of three groups.

Control group: incubated in standard bicarbonate medium

$ACTH_{4-10}$-7-D-phe (0.5) group: incubated in bicarbonate medium with $ACTH_{4-10}$-7-D-phe in a concentration of 0.5 µg/ml

$ACTH_{4-10}$-7-D-phe (1.0) group: incubated in bicarbonate medium with $ACTH_{4-10}$-7-D-phe in a concentration of 1.0 µg/ml.

All slices were preincubated for 10 min and incubated for 30 min after the addition of 1.25 µCi ^{14}C-leucine to the incubation medium. The protein was extracted and estimated as described before (Section 5 II A (ii)).

Results

$ACTH_{4-10}$-7-D-phe, in a concentration of 0.5 or 1 µg/ml, has no significant effect on the incorporation of ^{14}C-leucine into brain stem protein in vitro (table 14).

TABLE 14 : To show the effect of $ACTH_{4-10}$-7-D-phe on the incorporation of ^{14}C-leucine into rat brain stem protein in vitro

Group	Specific Activity (dpm/mg protein)
Control	1089.5 ± 271.4 (12)
$ACTH_{4-10}$-7-D-phe (0.5)	1094.0 ± 310.7 (6)
$ACTH_{4-10}$-7-D-phe (1.0)	1047.5 ± 295.4 (6)

Results expressed as mean specific activity ± standard deviation. (Number of slices)

Discussion

$ACTH_{4-10}$-7-D-phe has no effect on brain stem protein synthesis *in vitro*. This is in contrast with the results presented in Section 2 (vi) where this peptide tended to decrease the levels of protein labelled *in vivo*.

C. General Discussion

$ACTH_{4-10}$ increases the incorporation of ^{14}C-leucine into brain stem protein *in vitro*. This suggests that $ACTH_{4-10}$ acts directly on the brain rather than by a mediator from some other part of the body or by increasing the uptake of amino acids to the brain. As $ACTH_{4-10}$ has no effect on the uptake of leucine into the slice, it appears to change protein metabolism directly. Also as $ACTH_{4-10}$-7-D-phe has no effect on the incorporation of ^{14}C-leucine into protein *in vitro*, even though it has a similar amino acid composition to $ACTH_{4-10}$, one may assume that the stimulation produced by $ACTH_{4-10}$ is not due to an increase in free amino acids caused by the breakdown of the peptide, but rather by stimulation by the whole molecule.

The evidence that $ACTH_{4-10}$-7-D-phe has no effect on brain protein synthesis *in vitro* suggests that the effect of $ACTH_{4-10}$ on brain protein synthesis *in vitro* is not related to the behavioural effects of the peptide. $ACTH_{4-10}$ and $ACTH_{4-10}$-7-D-phe have opposite effects on behaviour (Greven and de Wied, 1973) so one might expect them to show opposite biochemical responses although it is possible that the behavioural tests are more sensitive than the biochemical tests.

Neither peptide had any effect on the incorporation of ^{14}C-leucine into retinal protein *in vitro* even though the retina has the same embryological origin as the brain. This suggests that $ACTH_{4-10}$ specifically acts on certain brain stem structures. This is also indicated by the experiments described in Section 2, where $ACTH_{4-10}$ stimulated the incorporation of leucine into brain stem protein while having no effect on the cortex or the liver, and by the implantation experiments of van Wimersma Greidanus and de Wied (1971) who showed that peptides were behaviourally active only when implanted in certain brain stem areas.

SECTION 6

TO SHOW THE EFFECT OF $ACTH_{4-10}$ ON Na^+,K^+-ATPase AND Mg^+ATPase IN RAT BRAIN.

Introduction

Certain inbred mice and rat strains are known to show different behavioural patterns, especially for learning and memory (Royce and Cavington, 1960; Sudak and Maas, 1964; Bovet et al., 1968). These patterns seem to be genetically determined (McLearn, 1965; Bovet et al., 1968; Oliverio et al., 1972). Bovet et al. (1968, 1969) and Oliverio et al. (1972) have demonstrated that the strain of mice DBA/2J show a high level of avoidance behaviour, while another strain, C57/BL6J, are rather poor at 'learning' an avoidance response. Various biochemical parameters have been investigated, in these two strains, in an attempt to detect any differences which would account for their different behaviour patterns. Differences in the cholinergic (Bovet et al., 1966; Ebel et al., 1973; Pryor et al., 1966), adrenergic (Ciaranello et al., 1972; Kempf et al., 1974) and serotoninergic (Valatx and Jouvet, 1971) systems have been reported. Recently Stefanovic (Stefanovic et al., 1974) has described differences in Na^+,K^+-ATPase of the cortex and hypothalamus. The DBA/2J mice, who are good at 'learning' a conditioned avoidance response, have significantly higher brain Na^+,K^+-ATPase activity than the C57BL/6J mice.

As $ACTH_{4-10}$ increases the ability of rats to acquire a conditioned avoidance response (de Wied, 1969), it was of

interest to see if this peptide had any effect on Na^+,K^+-ATPase. A positive effect would imply that the stimulation of Na^+,K^+-ATPase was involved in conditioned avoidance behaviour.

Method

Ten rats were randomly assigned to one of 2 groups:

$ACTH_{4-10}$ group received daily intraperitoneal injections of 0.2 ml of a neutral solution containing 20 µg $ACTH_{4-10}$.

Control group received daily intraperitoneal injections of 0.2 ml saline.

On the 14th day of the experiment all the rats were killed and Na^+,K^+-ATPase and Mg^{2+}ATPase was estimated in three brain areas. The areas selected were the pons, the hypothalamus and half the cortex, as defined by Stefanovic et al. (1974). The samples of brain were homogenized in distilled water (10 µl/1 mg wet tissue) and ATPase activity in aliquots of the homogenate was estimated by the method described in the Appendix. Protein in the samples was estimated by the method of Lowry (Lowry et al., 1951). Details of this method may also be found in the Appendix.

Results

$ACTH_{4-10}$ treatment has no significant effect on brain Na^+,K^+-ATPase (Table 1) or on Mg^{2+}-ATPase activity (Table 2). Mg^{2+}-ATPase activity is expressed as the ATPase activity remaining after the addition of 1mM-ouabain or the omission of Na^+ from the incubation medium Na^+,K^+-ATPase is represented by the ATPase activity which is inhibited by the omission of Na^+.

TABLE 1 : The effect of $ACTH_{4-10}$ on Na^+,K^+-ATPase activity in different rat brain regions

	Treatment Group	
	$ACTH_{4-10}$	Control
Cortex	9.864 ± 2.524	8.776 ± 3.264
Hypothalamus	13.616 ± 3.253	13.008 ± 4.294
Pons	11.230 ± 2.808	10.404 ± 3.490

Values are expressed as µmol P_i liberated/mg protein/hour.
Each value is the mean of 5 experiments ± standard deviation.

TABLE 2 : The effect of $ACTH_{4-10}$ on Mg^{2+}-ATPase activity in different rat brain regions

	Low N_a		Ovabain	
	$ACTH_{4-10}$	Control	$ACTH_{4-10}$	Control
Cortex	16.092±3.32	13.482±5.579	14.942±2.665	13.358±2.025
Hypothalamus	12.266±4.253	9.822±3.482	12.738±3.269	13.704±2.070
Pons	9.954±2.182	9.904±2.814	6.144±3.990	9.754±2.614

Values are expressed as uMol P_i liberated/mg protein/hour.
Each value is the mean of 5 experiments ± standard deviation.

Discussion

$ACTH_{4-10}$ treatment has no effect on ATPase activity in the brain cortex, hypothalamus or pons. This suggests that although activation of brain Na^+,K^+-ATPase may be involved in the acquisition of the conditioned avoidance response, ACTH peptides do not stimulate avoidance

acquisition through this system.

It is possible that any changes in the ATPase system caused by $ACTH_{4-10}$ would be small and might not be detected when the whole homogenate is studied. Attention was therefore focused on ATPase activity in the synaptosomes, the site where one would expect changes in Na^+,K^+-ATPase to be seen.

To show the effect of $ACTH_{4-10}$ treatment on Na^+,K^+-ATPase and Mg^{2+}-ATPase activity in synaptosomes prepared from rat brain stem.

Introduction

DBA/2J mice have higher brain acetyl cholinesterase, choline acetyl transferase and Na^+,K^+-ATPase activities than C57/BL6J mice (Ebel et al., 1973; Stefanovic et al., 1974). They are also able to acquire a conditioned avoidance response more quickly than C57/BL6J mice (Bovet et al., 1968; 1969). As it has been proposed that Na^+,K^+-ATPase is associated with nerve conduction and synaptic transmission (Abdel-Latif et al., 1970; Alfei and Venturini, 1972) and especially with the choline acetyl transferase system (Alfei and Venturini, 1972), it is possible that these differences in enzyme activities are linked and together produce differences in behaviour between the two strains of mice. One would therefore expect the changes in ATPase activity to be associated with the external synaptosomal membrane which is known to demonstrate high Na^+,K^+-ATPase activity (Hosie, 1963).

Method

Six, male albino Wistar rats were randomly assigned to one of 2 groups.

$ACTH_{4-10}$ group received daily intraperitoneal injections of 0.2 ml of a neutral solution containing 20 µg peptide.

Control group received daily intraperitoneal injections of 0.2 ml of physiological saline.

On the 14th day of the experiment all rats were killed and the brain stem (as defined by Gispen et al., 1972) removed. Synaptosomes were prepared from this fraction by the method described in Section 3 (ii). ATPase activity in the synaptosomal fraction was estimated by the method described in the Appendix.

Results

$ACTH_{4-10}$ has no effect on synaptosomal ATPase activity (table 3).

TABLE 3 : The effect of $ACTH_{4-10}$ on Na^+,K^+-ATPase and Mg^{2+}ATPase activity in rat brain synaptosomes

Group	Na^+,K^+-ATPase	Mg^{2+}ATPase (Low Na^+)	Mg^{2+}ATPase (Ouabain)
$ACTH_{4-10}$	14.95 ± 2.387	18.163 ± 1.606	19.230 ± 2.971
Control	18.043 ± 2.889	16.650 ± 1.271	16.046 ± 2.885

Values are expressed as uMol P_i liberated/mg protein/hour. Each value is the mean of 3 experiments ± standard deviation.

Discussion

$ACTH_{4-10}$ does not effect the conditioned avoidance response by a mechanism involving stimulation of brain ATPase.

SECTION 7

TO INVESTIGATE THE EFFECTS OF ACTH PEPTIDE ANALOGUES ON RAT BRAIN ELECTRICAL ACTIVITY

Introduction

In 1952 Torda and Wolff reported that single injections of ACTH (1-8 mg) into rats induced an increased brain electrical activity and lowered the convulsion threshold to pentamethylene tetrazol. Similar effects were noted in non-operated, hypophysectomized, adrenalectomized and sodium-injected, non-operated controls suggesting that this effect was not mediated by the adrenal cortex. Chronic injection of ACTH to intact rats for 3-4 days prior to recording increased the threshold to pentamethylene tetrazol and did not increase the brain electrical activity. In the same year, Woodbury reported that, after 28 daily injections of ACTH, the threshold to electroshock seizures was increased by 11%.

Torda and Wolff concluded that ACTH modifies the electrical activity of the brain by at least 2 mechanisms. A shift of electrolytes, including brain sodium was postulatedto explain the decrease in electrical activity following prolonged ACTH administration. As intact rats were used for this study it is probable that these effects are mediated by corticosteroids. A mechanism independent of the effect on the adrenal cortex was postulated to explain the hyperexcitability produced by single injections of ACTH.

The latter mechanism seemed to be confirmed by Sandman et al. (1971) who noted an increase in high voltage activity in the EEG following one injection of 20 µg porcine MSH. As MSH does not stimulate the adrenal cortex, this appears to be a direct effect on the brain. The authors compared the electrical activity after MSH with the pattern of hippocampal arousal and deduced that limbic structures might be involved in MSH-induced behaviour.

Wasserman et al. (1965) using young rats, failed to repeat the early work of Woodbury and Torda and Wolff, when he found that treatment with ACTH for one week reduced the threshold to minimal electroshock seizures in intact and adrenalectomized rats. The reduction in seizure threshold was correlated with an increase in the concentration of intracellular sodium in the brain. This seemed to be independent of the stimulation of the adrenal glands. There is much evidence that high sodium concentrations in the brain have convulsant effects. Sawyer and Gernant (1956) and Glaser (1964) found that intraventricular injections of hypertonic saline induced seizure activity whereas other hypertonic solutions did not. An increase in intracellular sodium in the brain has been demonstrated in animals with heightened seizure susceptibility caused by electrical stimulation, pentylentetrazol, withdrawal of anaesthetizing doses of CO_2 and adrenalectomy.

The differing results reported in animal studies could be due to differences in the number and size of the doses of ACTH given or could be due to the variety of ages of animal studied.

Contradictions are also found in the clinical studies. ACTH was first reported to have anticonvulsant effects in 1950 (Klein and Livingston, 1950). It is more effective in children than adolescents (Millichap and Bickford, 1962) whereas in young rats ACTH appears to reduce the seizure threshold to electroshock seizures. Whether the ACTH action is mediated via the adrenal cortex is still not certain. In one trial several patients benefitted from ACTH while cortisone had no effect (Millichap and Jones, 1964) but the use of corticosteroids has been advocated in the treatment of myoclonic spasms, (Low, 1958).

Obviously there is a great deal of disagreement in the literature as to the mode of action of ACTH on seizure thresholds in rats andin epilepsy in humans. ACTH peptide analogues could be a useful tool in unravelling some of the problems. $ACTH_{4-10}$ has similar properties to ACTH in its actions on behaviour (de Wied, 1969) but does not stimulate the production of corticosteroids. The use of $ACTH_{4-10}$ could therefore give some indication as to whether ACTH alters brain electrical activity directly or via the adrenal cortex. If it acts by the former mechanism $ACTH_{4-10}$ would be of obvious clinical significance.

In 1960 Kopeloff reported that cobalt has an epiloptogenic effect when applied to the brains of mice and rats (Kopeloff, 1960). This was confirmed by the electroencephalographic studies of Dow et al. (1962). Fischer et al (1967) refined the technique of cobalt implantation so as to produce a discrete lesion and a delay between

cortical insult and the development of the focus. Dow et al. (1972) used this technique and devised a method for chronic recording of the electrocorticogram (E Co G) in the conscious rat. This was the technique used to study the effect of $ACTH_{4-10}$ on brain electrical activity.

A. General Method

The method of operation of the rats and recording was based on that of Dow et al. (1972) and the method of analysis of the record on that of Hill and Townsend (1973).

Male, Piebald Virol Glaxo (PVG) rats weighing 200-250 gm (approx. 2½ months old) were used throughout, these rats were chosen as they are relatively resistant to respiratory disorders.

(i) Operation

All surgical procedures were carried out under aseptic conditions.

The rat was placed in a plastic sandwich box where it was subjected to 11% halothane in oxygen (2.5L/min). After it had become anaesthetized, it was removed from the box and its head shaved, and sterilized with iodine. The rat was placed in a sterotatic frame and 7-9% halothane was applied through a head mask throughout the rest of the operation. The skull was exposed by making a curved, cranial incision (2-3 cm long) close to the right eye. The skull was cleared and the naso-frontal, coronal, lamboidal and sagittal sutures located. These sutures were used as reference points to ensure that the electrodes were placed

over the same brain area in each rat. Four holes were drilled in the skull as in fig. 1, 3 mm from the sagittal and coronal sutures. The holes were made with a dental drill (Renda, Model RA/21) and a round No. 6 bur. Care was taken not to go through the underlying dura. The skull was then wiped clean of any bone dust and blood, although there was very little of the latter. In rats that were to receive a cobalt implant (Section 6C) hole b (fig.1) over the frontal cortex was prepared for the cobalt. The dura was split with the end of a sterile 23-gauge needle. Cobalt-gelatine had previously been prepared by the method of Fisher et al. (1967). A film 1 mm thick was stored on a microscope slide in 80% ethanol. Immediately before the experiment a cylinder of 1 mm diameter was cut from the slide using a 'microslicer'. Using a pair of fine forceps, this cylinder of cobalt was inserted perpendicularly into the cortex so that its top was flush with the cortical surface. Stainless steel screws, especially constructed in our department workshop, were then screwed into the skull. The design of the screws and their position in the skull is illustrated in fig. 2. It was necessary to secure the screws into the skull with a little cold-curing acrylic resin (Simplex) which was applied on the tip of a 23 gauge needle. When all the screws were secure, the whole area was sprayed with antibiotic (Polybactrin, Calmic Ltd.). Holes were punched in the skin flap so that the flap fitted neatly over the electrodes and allowed them to protrude externally. The incision was then closed with

Fig. 1.

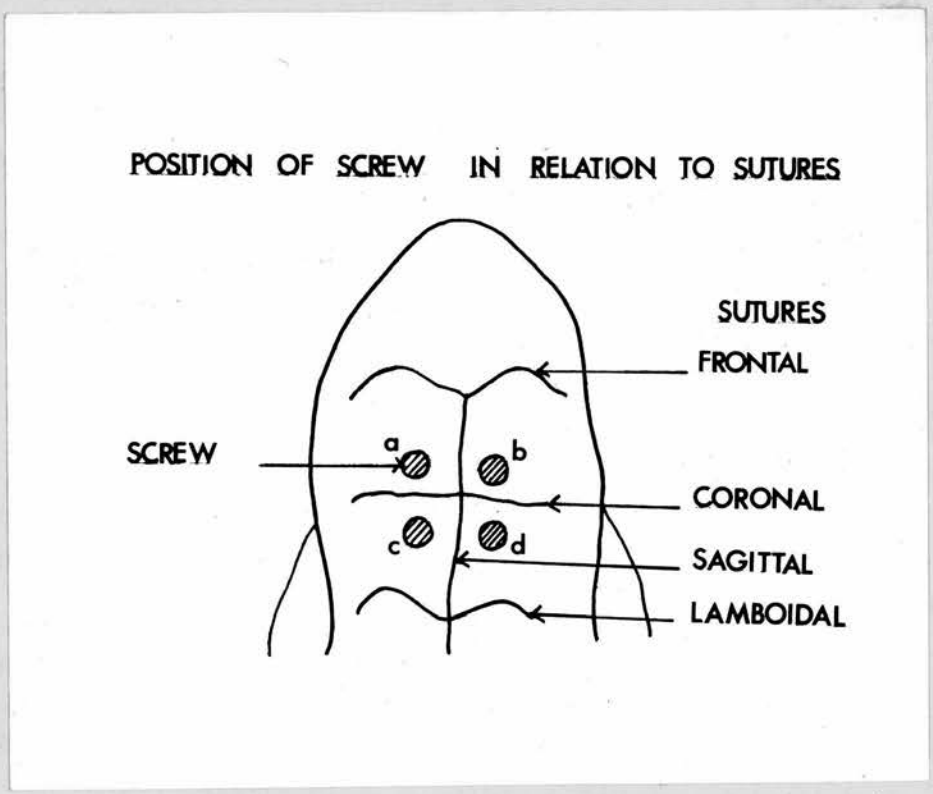
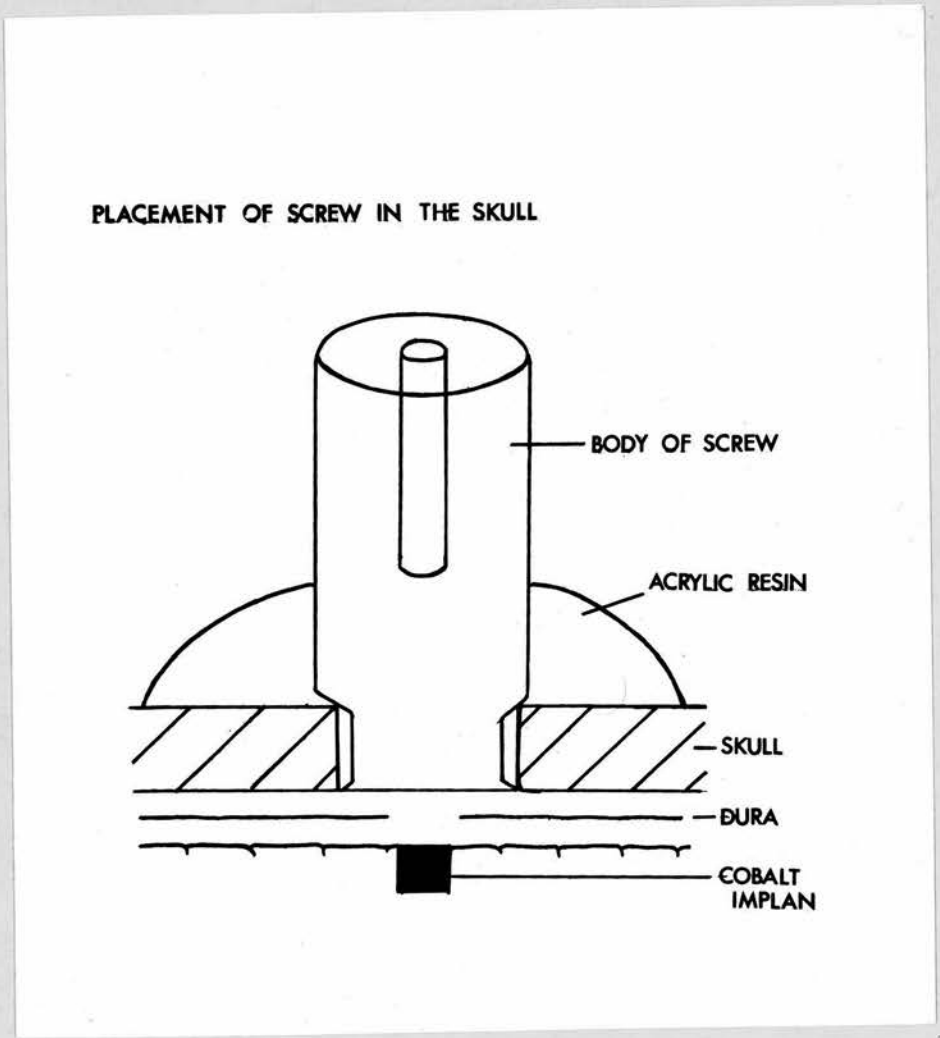

POSITION OF SCREW IN RELATION TO SUTURES
SUTURES
FRONTAL
SCREW
a
b
CORONAL
c
d
SAGITTAL
LAMBOIDAL

Fig. 2.


PLACEMENT OF SCREW IN THE SKULL
BODY OF SCREW
ACRYLIC RESIN
SKULL
DURA
COBALT IMPLAN

Michel clips (Aesculap, 12 x 3 mm). The whole procedure took between 10 and 20 min.

No special post operative care was found necessary. The skin wound healed within 7 days and the Michel clips could then be removed. Normally there was an almost 100% success rate in the operation, the rats remaining healthy for several months after the operation. All the screws remained firm throughout the experiments.

(ii) Recording

ECoG (Electrocorticogram) recordings could be made from the unrestrained conscious rat within 24 hr after the operation. Gold spring connectors attached to wire (Grass) were fitted into the hollow screws. These allowed the brain potentials to be recorded on a Grass Model 7 polygraph and onto magnetic tape using a Tandberg-100 tape recorder. After fitting the connectors the rat was placed in a perspex-sided cage and allowed 10 min to adjust to its new surroundings. It was permitted to move around the cage freely but usually after the first few minutes, the rat sat quietly. Controls on the Grass polygraph were set as recommended for recording the human EEC. Potentials from the right (b) and d) and left (a and c) sides were recorded on separate channels of the recorder. After the rat had become accustomed to its new surroundings, a 30 sec. calibration wave was recorded. This was a 100 microvolt square wave pulse. The rat brain potentials were then recorded for 10 min unless otherwise stated in the experiment. The rats were studied throughout the

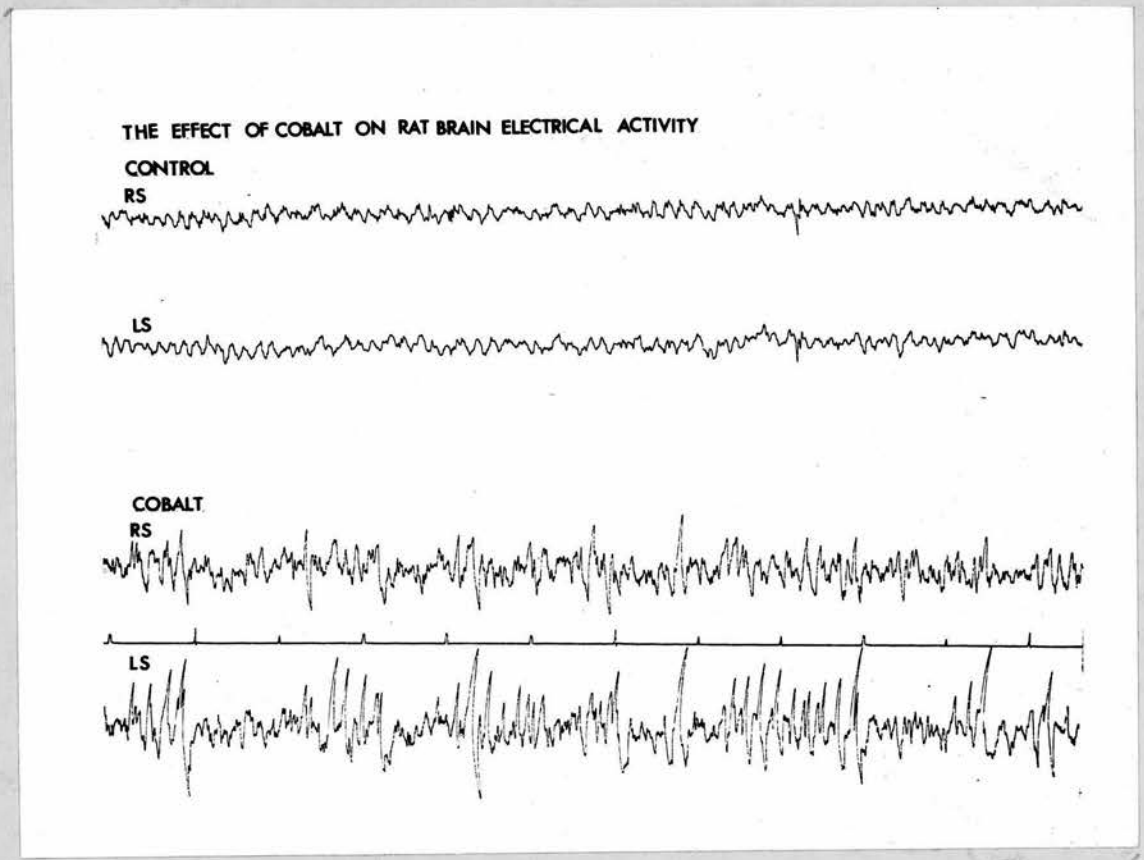
experiment for any signs of twitching. Any abnormal behaviour was marked on the polygraph record.

(iii) Interpretation of the records

A subjective assessment of the polygraph record was made indicating the presence of spikes, polyspikes and slow wave. In addition the ECoG record on magnetic tape was analysed by a real time computer program which estimates the degree of spiking using a criterion of "sharpness" (Hill and Townsend, 1973). This analysis was carried out by Dr. G. Hill at the Western General Hospital, Edinburgh.

A typical polygraph trace from a rat without a cobalt implant and from a rat 10 days after a cobalt implant in fig. 3. The traces from animals without cobalt remained unaltered throughout the weeks when recordings were made. In rats with a cobalt implant in the right frontal cortex, single spikes and polyspikes were seen in the trace from the right (primary focus) side and the left (secondary or mirror focus) side 2-4 days after the implantation. The number of spikes/min recorded increased daily reaching a maximum 9 or 10 days after implantation. At the same time most rats developed a whisker and left forelimb twitch which was associated with a spike discharge. The degree of spiking decreased giving a normal trace at approximately 28 days post-op. Throughout there were fewer spikes on the primary side than on the secondary lesion side. Fig. 4 shows the development of spiking in rats with a cobalt implant in the right frontal cortex. The number of spikes/min are as calculated by the computer analysis from the magnetic tape record.

Fig. 3.



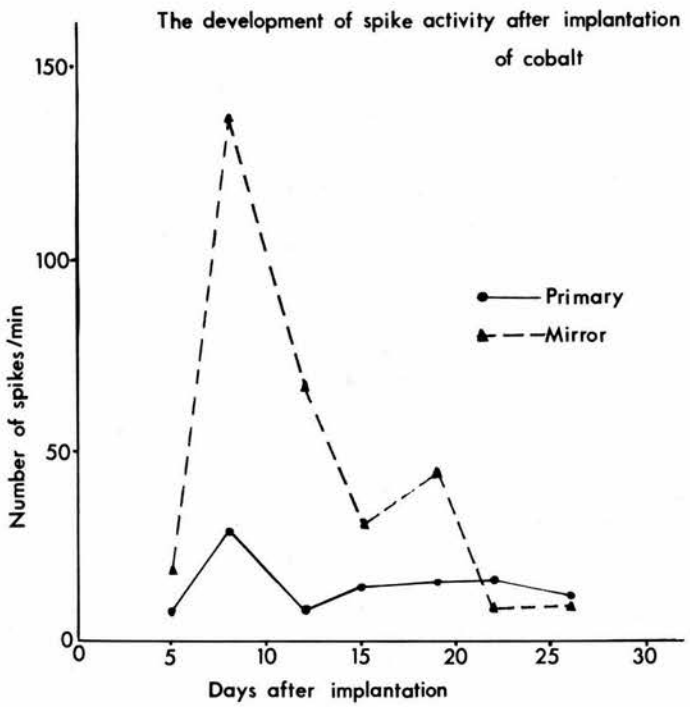
RS = Right side

LS = Left side

—— 1 sec

| 200 microvolt

Fig. 4.


The development of spike activity after implantation of cobalt
Number of spikes/min
150
100
50
0
0
5
10
15
20
25
30
Days after implantation
Primary
Mirror

B. To show the effect of $ACTH_{4-10}$ on the brain electrical activity of an intact rat

Method

Four recording screws were fixed into the skull of 3 rats by the method described above (Section 6 A). Cobalt was not implanted in the brain. Brain electrical activity was recorded on the 8th and 28th day after the operation (post-op). Each rat was used as its own control in that a recording was made before the administration of drug, after a control injection of saline and after $ACTH_{4-10}$. 15 min recordings were made for the control periods and 2 hour after $ACTH_{4-10}$. Each rat received a different dose of peptide. Rat one received 20 µg, rat two 100 µg and rat three 500 µg. All the rats were approximately 260 gm in weight. Recordings were made as described above.

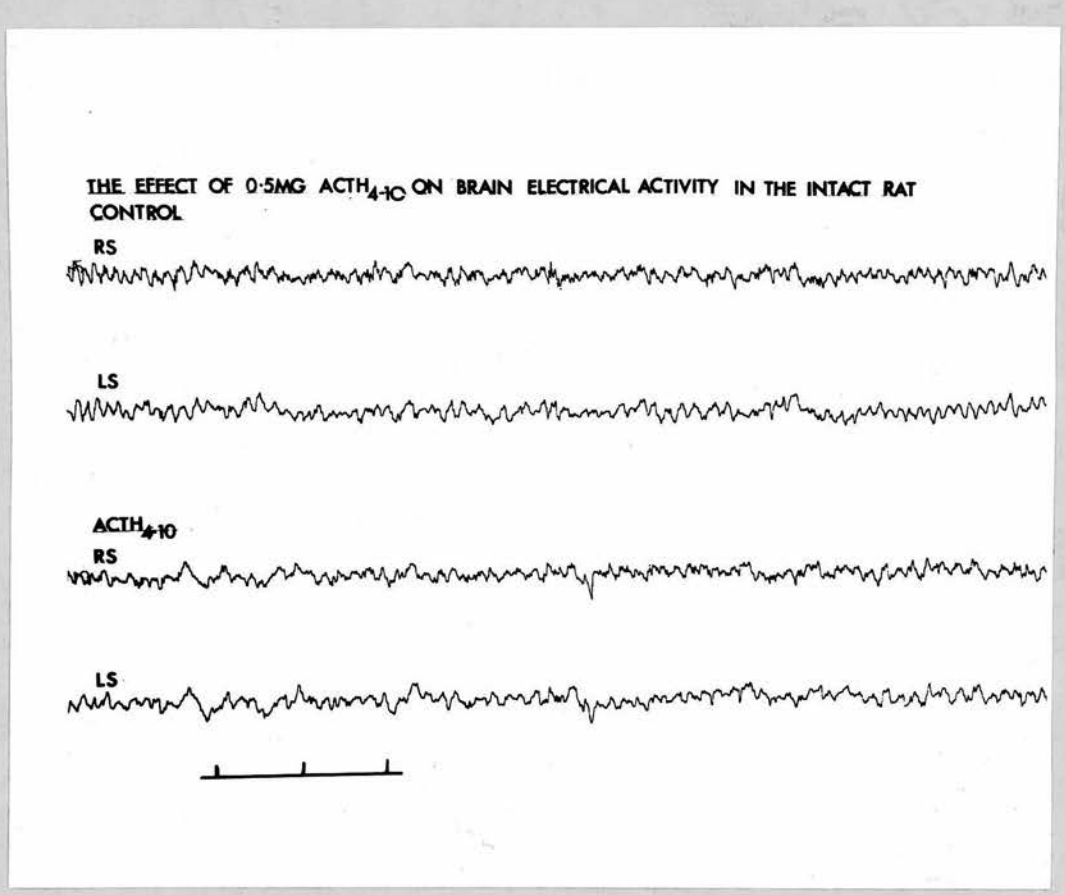
Results

No abnormal electrical activity was seen in any of the rats. That is there was no evidence of spiking or increased electrical activity either before or after the administration of peptide. Fig. 5 shows part of the polygraph trace before and after the administration of 0.5 mg $ACTH_{4-10}$. These traces are typical of all the recordings.

Discussion

It is apparent that an acute injection of $ACTH_{4-10}$ has no effect on brain electrical activity. These results may be compared with those of Torda and Wolff (1952) and Sandman et al. (1971) who found that acute injections of

Fig. 5.



RS = Right side

LS = Left side

—— 1 sec

| 200 microvolts

ACTH and MSH increased brain electrical activity. It seems probable that a larger part of the ACTH molecule than $ACTH_{4-10}$ is involved in this stimulatory effect.

C. To show the effect of $ACTH_{4-10}$ on the development of spike activity following cobalt implantation

Method

Eight male, PVG rats weighing 240-260 gm received an implant of cobalt in the right frontal cortex as described above. Four screws were fixed in the skull. The rats were assigned to one of two groups.

Control group received daily IP injections of 0.2 ml physiological saline.

$ACTH_{4-10}$ group received daily IP injections of 20 µg $ACTH_{4-10}$ in 0.2 ml saline.

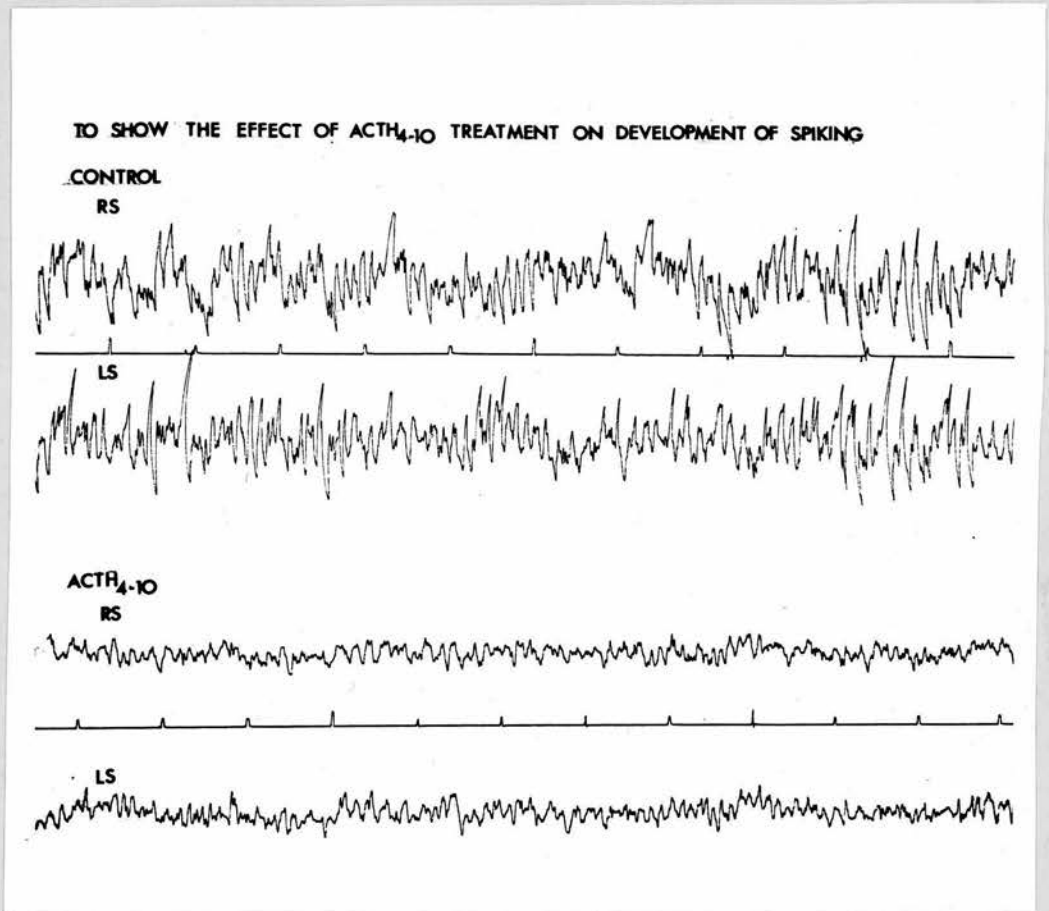
These injections started on the day of the operation and were continued throughout the experiment. ECoG recordings were made 2 times a week for 4 weeks.

Results

$ACTH_{4-10}$ treated animals showed a marked decrease in electrical activity in the ECoG compared to control animals (fig. 6). There was pronounced flattening of the trace from both sides of the brain but this tended to be more pronounced in the right, primary focus, side.

$ACTH_{4-10}$ significantly reduced the number of spikes/min induced by cobalt and the animals appeared to recover rather more quickly than the controls (fig. 7 and table 1).

Fig. 6.



RS = Right side

LS = Left side

Time marker: 1 sec | 200 microvolts

Fig. 7.

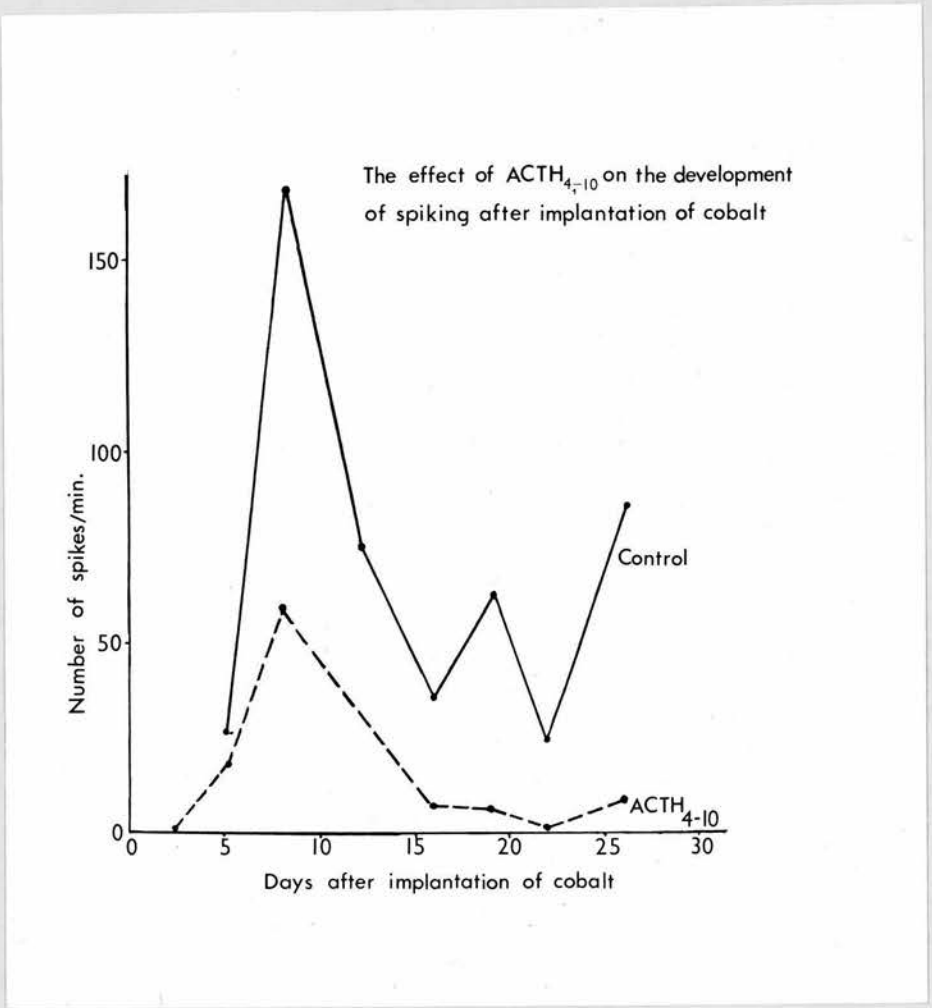

The effect of ACTH4-10 on the development of spiking after implantation of cobalt
Number of spikes/min.
150
100
50
0
0
5
10
15
20
25
30
Days after implantation of cobalt
Control
ACTH4-10

TABLE 1 : To show the number of spikes/min recorded in the ECoG at varying times after implantation of cobalt

5 dpo			
Control	26.75 ± 24.06	(4)	t = 0.56
Peptide	19.5 ± 8.1	(4)	N.S.
8 dpo			
Control	166.7 ± 28.8	(3)	t = 2.33
Peptide	63.3 ± 59.7	(4)	N.S.
12 dpo			
Control	75.3 ± 32.91	(3)	t = 1.95
Peptide	30.8 ± 17.3	(4)	N.S.
15 dpo			
Control	42.3 ± 11.14	(3)	t = 4.41
Peptide	8.0 ± 6.04	(4)	P < .01
19 dpo			
Control	60.33 ± 35.9	(3)	t = 2.44
Peptide	7.75 ± 49.1	(4)	N.S.
22 dpo			
Control	23.3 ± 6.6	(3)	t = 4.4
Peptide	3.75 ± 3.0	(4)	P<0.01
26 dpo			
Control	88.7 ± 21.5	(3)	t = 4.86
Peptide	12.0 ± 13.6	(4)	P < 0.01

The results are expressed as mean spikes/min ± standard deviation (Number of rats)

The statistical analysis is by the students t-test with Bessel's correction.

If the degree of spiking in the two sides of the brain is considered separately (table 2), it may be seen that in the $ACTH_{4-10}$ treated animals few spikes were recorded from the primary lesion side. As values below 10 spikes/min are usually due to computer inaccuracies, it seems that spiking was only recorded from the mirror focus and these were below 10/min after 15 days.

Discussion

$ACTH_{4-10}$ treatment reduced the spike activity induced by a cobalt implant and reduced the overall levels of electrical activity suggesting that it is effective in inhibiting the development of an epileptic focus. There are several methods by which it could do this. For example, it could alter the ion balance of the nerve cells, possibly by reducing intracellular sodium; it could reduce the activity of stimulatory transmitters like glutamate or increase the activity of inhibitory transmitters like GABA; or it could stimulate regeneration of the nervous tissue around the cobalt implant. Although it would seem that the time course of the effect is too short to permit the last mechanism there is some evidence that ACTH does stimulate regeneration. Fertig et al. (1971) showed that ACTH stimulated axonal regeneration following stab wounds in rat brain and Shapiro et al. (1968) demonstrated that ACTH causes a significant elongation of motor end plates and increased branching of preterminal motor fibres at the rabbit neuromuscular junction. It is of interest that after cobalt implantation in the frontal cortex there is considerable

TABLE 2 : To show the number of spikes/min recorded in the ECoG at varying times after implantation of cobalt

	Left side, Mirror focus		Right side, Primary focus	
2 dpo				
Peptide	0.5 ± 0.87(4)		1.5 ± 1.5 (4)	
5 dpo				
Control	18.75±18.16(4)	t=0.17	8.0 ± 7.6 (4)	t=1.4
Peptide	16.75+ 6.80(4)	N.S.	1.75± 9.82(4)	N.S.
8 dpo				
Control	137.3 ±28.4 (3)	t=1.85	29.3 ± 7.1 (3)	t=5.14
Peptide	58.5 ±57.0 (4)	N.S.	4.75± 3.26(4)	P<0.005
12 dpo				
Control	67.7 ±28.3 (3)	t=2.14	7.66± 4.64(3)	t=1.28
Peptide	25.0 ±15.6 (4)	N.S.	3.5 ± 2.5 (4)	N.S.
15 dpo				
Control	31.7 ±13.5 (3)	t=2.67	14.0 ± 9.27(3)	t=2.45
Peptide	7.5 ± 6.1 (4)	P<0.05	0.5 ± 0.5 (4)	N.S.
19 dpo				
Control	45.3 ±33.2 (3)	t=1.94	15.0 ±10.0 (3)	t=2.29
Peptide	6.5 ± 5.54(4)	N.S.	1.25± 1.29(4)	N.S .
22 dpo				
Control	7.66± 3.29(3)	t=1.62	15.7 ± 3.29(3)	t=7.33
Peptide	3.0 ± 3.08(4)	N.S.	0.75± 0.82(4)	P<0.001
26 dpo				
Control	77.0 ±24.8 (3)	t=3.99	11.66± 5.55(3)	t=2.84
Peptide	10.5 ±11.5 (4)	P<0.025	1.5 ± 2.06(4)	N.S.

The results are expressed as mean spikes/min ± standard deviation (Number of rats).

The students t-test with Bessel's correction was used for the statistical analysis of the results.

degeneration of the thalamic area around the nucleus parafascicularis on the primary focus side (Emson and Joseph, 1974). As this area has been implicated in the behavioural activity of ACTH peptides (Bohus and de Wied, 1967a, 1967b) it is conceivable that $ACTH_{4-10}$ acts in this area either by stimulating the remaining neurons or by reducing the degeneration, thereby restoring activity towards normal levels.

GENERAL DISCUSSION

$ACTH_{4-10}$ treatment increases the incorporation of ^{14}C-leucine into rat brain protein *in vivo* (Section 2). The effect is first visible 1 hour after administration of labelled precursor. This information from the intact rat may be compared to that from the hypophysectomized rat. Here $ACTH_{1-10}$ has been shown to increase the incorporation of ^{3}H-leucine into rapidly turning over protein (Schotman et al., 1972). That is, in the hypophysectomized rat an effect is visible 2-15 min after administration of precursor. In both cases, the changes seem to be mainly confined to the brain stem area as defined by Gispen et al. (1972) and the 7-D-phe isomer tends to have the opposite effect although this is rather more marked when hypophysectomized rats are used.

There could be several possible explanations for the differences in the time course of the effect of ACTH peptides on protein metabolism in intact and hypophysectomized rats. The differences are not due to differences in the mode of administration of peptide (Section 2 (ii)) but

could be due to different activity of the peptides used. Schotman et al. (1972) used $ACTH_{1-10}$ whereas $ACTH_{4-10}$ was used here. Both peptides have quantitatively similar effects on behaviour (de Wied, 1969) so one might expect them to have similar effects on biochemical parameters, but it is possible that brain protein metabolism is more sensitive to $ACTH_{1-10}$ than $ACTH_{4-10}$. It was not possible to test this hypothesis as $ACTH_{1-10}$ was not available. It is possible that ACTH peptides act by different mechanisms in hypophysectomized and intact rats with peptides effecting protein with a short half-life in the former and a longer half-life in the latter. Although the hypophysectomized animal is subject to serious metabolic derangements caused by the hormonal imbalance it seems unlikely, although not impossible, that it would respond by a different mechanism to that of the intact rat. A more probable explanation is that the hypophysectomized rat is more sensitive than the intact rat to factors change protein metabolism because of its already reduced rate of protein synthesis (Dunn and Korner, 1966; Takarishi et al., 1970). One might expect that the reduced synthetic rate in the hypophysectomized rat would be more easily increased than the normal synthetic rate in the intact rat where homeostatic mechanisms have to be overcome. If this is true, the changes seen in the hypophysectomized rat would be larger and therefore more easily detected than in the intact rat. It is likely that any changes in the intact rat would only be visible at the time of maximum incorporation (1-4 hr) the time when, in

fact, increases in incorporation were detected in the brain stem.

The increase in incorporation of ^{14}C-leucine into protein could be produced by various mechanisms. $ACTH_{4-10}$ could effect nucleic acid metabolism. As ACTH peptides have no effect on polysome profiles (Gispen and Schotman, 1970), RNA polymerase (Dewar, 1972) or RNA synthesis (Reading and Dewar, 1971; Schotman et al., 1972) it seems that they do not act by this mechanism. ACTH peptides could reduce protein breakdown. Although this mechanism could explain the changes seen in the intact rat, the time course would be too long to explain changes seen in the hypophysectomized rat. Peptide treatment could stimulate protein synthesis by increasing the free pool of amino acids available either by mobilizing amino acids from some source in the body or by increasing the transport of amino acids into the brain. As $ACTH_{4-10}$ has no effect on the levels of free amino acids in plasma or brain (Section 4), this mechanism of action seems unlikely although a change in one small brain area cannot be ruled out at present. A direct effect of the peptides at the translational level is the last possible mode of action. $ACTH_{4-10}$ stimulates the incorporation of ^{14}C-leucine into brain stem slices while having no effect on the free pool of amino acids (Section 5). This supports the evidence that $ACTH_{4-10}$ does not effect the transport of amino acids to the site of protein synthesis. As the peptides have no effect on nucleic acid metabolism, it seems probable that they act at the translational stage of protein synthesis.

This possible mechanism has a parallel in the action of ACTH on the adrenal cortex. Protein synthesis but not RNA synthesis is necessary for ACTH to stimulate steroid production. Protein synthesis inhibitors (e.g. puromycin, cyclohexamide) block the stimulatory effect of ACTH *in vitro* (Ferguson, 1963) and *in vivo* (Garren et al., 1965) while the RNA synthesis inhibitor, actinomycin D has no effect *in vitro* (Ferguson and Morita, 1964) or *in vivo* (Garren et al., 1965; Ney et al., 1966) suggesting that ACTH is exerting its control at the translational level. In 1957, Haynes and Barthet showed that ACTH stimulates the formation of cyclic AMP in the adrenal gland and in 1959 Haynes et al. showed that cyclic AMP itself could induce steroidogenesis. This stimulation of steroidogenesis by cyclic AMP requires protein synthesis but not RNA synthesis (Ferguson, 1963; Grahame-Smith et al., 1967). The cyclic AMP receptor in the adrenal cell appears to be a protein kinase (Gill and Garren, 1970). Cyclic AMP activates this protein kinase by causing the dissociation of an inhibitor-receptor molecule from the enzyme moiety. Garren et al. (1971) postulate that ACTH binds to the adrenal cortical cell plasma membrane, stimulating adenyl cyclase to form cyclic AMP. Cyclic AMP, in turn, activates the protein kinase which then catalyzes the transfer of phosphate from ATP to ribosomes, thus regulating protein synthesis.

As yet, there is no evidence that ACTH or ACTH peptides modify protein synthesis in the brain by this mechanism. In isolated adrenal cells it has been shown that $ACTH_{4-10}$

is the smallest peptide which can stimulate steroid production (Schwyzer et al., 1971) and cyclic AMP production (Seeling and Sayers, 1972) although it is considerably less active than ACTH. Seeling and Sayers (1972) have postulated that this peptide sequence is the active centre of the ACTH molecule for this function. It is conceivable that the ACTH receptor would be similar in the brain, although as $ACTH_{4-10}$ does not stimulate steroid production *in vivo* one must postulate that the brain is rather more sensitive to its action.

Cyclic AMP has been shown to stimulate endogenous protein kinase phosphorylation of microsomes isolated from ox brain (Weller and Rodnight, 1970) and ribosomal protein from bovine anterior pituitary gland (Barden and Labrie, 1973) but neither Forn and Krishna (1970) nor Burkhard and Gey (1968) found an increase in brain cyclic AMP levels after ACTH stimulation. These experiments do not exclude the possibility that ACTH and ACTH peptides stimulate brain adenyl cyclase as it might be expected that any changes would be specific to a small brain area and would not be of large magnitude.

It should be noted that one of the advantages of control of protein synthesis at the translational level is that this is a quick and sensitive control mechanism. In the adrenal cortex changes in protein synthesis are seen a few minutes after ACTH stimulation and stop almost immediately after removal of ACTH (Garren et al., 1971). This mechanism would account for the quick response seen by

Schotman (Schotman et al., 1972) in the hypophysectomized rat, but would seem unnecessary for the slower response seen in my experiments using the intact rat (Section 2) unless one postulates that in the intact rat we are only observing the later part of the response.

Some indication as to the significance of ACTH and peptide action on the brain may be obtained from a study of the areas of brain involved. Both in the intact and hypophysectomized rat, ACTH peptides increase the incorporation of leucine into the brain stem areas described by Gispen et al. (1972). This area has also been implicated in the behavioural effect of the peptides. When $ACTH_{1-10}$ is implanted in the rostral mesencephalon or the caudal diencephalon at the posterior thalamic level, it delays the extinction of a conditioned avoidance response in a manner similar to that of systemic administration (Van Wimersma Greidanus and de Wied, 1969, 1971). When implated into other sites the peptide was ineffective. Bilateral destruction of the nucleus parafascicularis facilitates extinction of the conditioned avoidance response (Bohus and de Wied, 1967a). In rats bearing lesions in this nucleus MSH was unable to affect extinction in amounts that caused resistance to extinction in intact rats (Bohus and de Wied, 1967b). These results suggest that the nucleus parafascicularis is implicated in the behavioural effect of ACTH analogues. The nonspecific thalamic reticular area is thought to have an important integrative function because all incoming information converges on this

structure (Cardo, 1965, 1967). As the studies described above indicate that ACTH peptides act in this region it is possible that they modulate neuronal transmission at this point of sensory integration. Changes in protein metabolism also appear to occur in this general area. It is possible that ACTH and ACTH peptides stimulate cell metabolism facilitating the formation of new synaptic connections in this area.

For the possible physiological significance of peptide action one must consider the role of ACTH. As ACTH is secreted in large quantities in response to stressful situations one might expect that it effects behaviour associated with stress. In 1970, Weiss et al. postulated that ACTH increases excitibility which leads to an increase in generalized fear or anxiety in fear situations and various electrophysiological studies indicate that ACTH and ACTH analogues have a central excitatory effect (Sawyer et al., 1968; Steiner, 1970; Pfaff et al., 1971; Sandman et al., 1971). This was not supported by the data described in Section 7 where changes in the ECoG were measured but it is probable that this technique would not be sufficiently sensitive to detect changes in hypothalamic neurons that may be detected when recordings are taken from single neurons (Steiner, 1970).

In summary, $ACTH_{4-10}$ increases protein synthesis in certain brain stem areas possibly by a mechanism involving control at the translational level. These changes are probably related to the effect of ACTH peptides on conditioned avoidance behaviour.

APPENDIX

(A) Method of estimation of protein

The method used was that of Lowry (Lowry et al., 1951). This method is based on the formation of a blue colour on the reaction of tyrosine residues with Folin Coicalteau reagent under alkaline conditions.

Reagents

A. 2% Na_2CO_3 in 0.1N NaOH.

B_1. 1% $CuSO_4 . 7H_2O$.

B_2. 2% Na/K tartrate.

B. 1 part B_1: 1 part B_2.

C. 50 parts A and 1 part B.

D. Folin Coicalteau reagent diluted 1:1:5 with distilled deionized water.

Procedure

Solutions B and C were prepared just before use. 3 ml of solution C was mixed with 0.3 ml protein solution and then allowed to stand at room temperature for 15 min. 0.3 ml of solution D was added and the solution mixed immediately. This mixture was allowed to stand at room temperature for 30 min and was then read at 750 nm, against a reagent/water blank, in an SP500 spectrophotometer.

Standards of bovine serum albumin, to give a final concentration of 5-25 µg/ml were included in each experiment.

(B) Method of estimation of DNA

(a) The method used was based on that of Schneider

(Schneider, 1957). Deoxyribose reacts with diphenylamine in acid solution to form a blue colour which may be detected spectrophotometrically. The method is sensitive in the concentration range of 0.025 to 0.4 mg DNA.

Reagent

1 gm diphenylamine

100 ml glacial acetic acid

2.75 ml concentrated sulphuric acid.

Procedure

0.5 ml of sample was mixed with 1 ml of reagent and this was boiled for 15 min. The mixture was then cooled and read at 600 nm against a reagent/water blank in an SP500 spectrophotometer (Pye-Unicam).

Standards of calf thymus DNA in the concentration range 0.025-0.4 mg were included in each experiment.

(b) In situations where a more sensitive method was required for the estimation of DNA, Burton's modification of Schneider's method was used (Burton, 1956). This method is sensitive in the range 0.004 to 0.02 mg DNA.

Reagent

1.5 gm diphenylamine

1.5 ml concentrated sulphuric acid

100 ml glacial acetic acid

0.5 ml of 16 mg/ml acetaldehyde was added just before the reagent was used.

Procedure

0.5 ml of sample was mixed with 1 ml of reagent. This was incubated at 30°C overnight (16 hr), cooled and read at 600 nm against a reagent/water blank. Standards of calf thymus DNA in the concentration range of 0.005 to 0.02 mg were included in each experiment.

(C) Estimation of Fumarase

The estimation is based on that of Koelle (1951). Malic acid is incubated with the enzyme and the formation of the α -double band of fumaric acid is observed spectrophotometrically.

Reagents

A. 0.536 gm L-malic acid

B. 0.1 M Na/PO_4 buffer, pH 7.4.

C. Distilled water.

D. 1N NaOH.

E. Mix reagent A with 60 ml of reagent B and 40 ml of reagent C. Bring to pH 7.4 with D and then dilute to a final volume of 120 ml with reagent C. Prepare reagent E immediately before use.

Procedure

The enzyme was activated by the addition of Triton X-100 to the enzyme suspension to give a final concentration of 1% Triton. The spectrophotometer cuvet carriage was prewarmed to 37°C. 3 ml of freshly prepared reagent E and 0.2 ml of the enzyme were mixed in a cuvet. The

optical density at 250 nm was measured for 1 to 10 min against an enzyme water blank.

(D) Estimation of Lactate Dehydrogenase

The method is based on that of Johnson (1960). The oxidation of $NADH_2$ is observed spectrophotometrically.

Reagents

A. 0.05M Tris buffer, pH 7.4.

B. Mix 50 ml of reagent A with 3.5 mg of $NADH_2$ and 2 mg of sodium pyruvate immediately before use.

Procedure

The enzyme was activated by the addition of Triton X-100 to the enzyme suspension to give a final concentration of 1% Triton. This releases occluded enzyme and inhibits utilization of $NADH_2$ via other systems. 3 ml of reagent B was then mixed with an appropriate volume of tissue suspension (usually 100 µl) in a cuvet. This was read at 340 nm against a water/enzyme blank for 1 to 10 min.

A change of extinction of1 is equivalent to 0.48 µmoles of NADH oxidized.

(E) Method for estimation of Na^+-K^+-ATPase and Mg^{2+}ATPase

The method used is based on that of Kurokawa et al. (1965). The enzyme is incubated with ATP and the ortho-phosphate released is measured by the method of Lindberg and Ernster (1956) described below.

Incubation Medium

	Final Concentration (mM)	Volume per tube (ml)
$MgCl_2$	6	0.5
KCl	5	0.5
NaCl	100	0.5
Tris-HCl buffer pH 7.4	30	0.6
EDTA in Tris	1	0.1

0.2 ml of tissue suspension

Procedure

The above incubation mixture was prepared in tubes in an ice bath and was then shaken at 37.5°C for 10 min. The reaction was started by the addition of 0.1 ml Tris-ATP to give a final concentration of 3mM and the incubation was continued for a further 15 min or 1 hr. The incubation time was determined by the predicted enzyme concentration. The reaction was stopped by the addition of 1 ml of ice cold 24% TCA. The reaction tubes were then left in ice for 10-20 min to allow complete precipitation of protein. The tubes were then spun at 5,000 rpm and aliquots of the supernatant were taken for the estimation of orthophosphate.

This procedure will give an estimate of total ATPase activity. Na^+-K^+-ATPase may be defined as the ATPase activity which is inhibited by the omission of Na^+ or by the addition of 1 mM ouabain while Mg^{2+}ATPase may be defined as the activity left after omission of Na or addition of 1mM ouabain. In all cases 3 tubes were used per estimation.

(i) containing the complete medium as described above

(ii) containing all the components of the medium except NaCl (soln) which was replaced by an equal volume of water

(iii) containing all the components of the medium but containing ouabain in the Tris buffer to give a final concentration of 1 mM ouabain.

In addition each estimation was carried out in triplicate. Appropriate blanks were included to correct for spontaneous hydrolysis of ATP and endogenous P_i in the brain homogenate.

Determination of Organic Phosphorous Compounds by Phosphate Analysis

The Method is based on that of Berenblum and Chain (1938) as modified by Martin and Doty (1949). The procedure used was that described by Lindberg and Ernster (1956).

Reagents

A. 5M H_2SO_4

B. 10% Ammonium Molybdate

C. Isobutanol-benzene mixture (1:1)

D. 10% $SnCl_2$ H_2O in conc. HCl. Freshly diluted 200 times with 0.5M H_2SO_4.

E. 3.2% v/v sulphuric acid in absolute ethanol.

Procedure

3 ml of the deproteinized sample containing between 0.05 and 1.0 µM phosphate was mixed in a test tube with 0.5 ml of A, 5 ml of C and 0.5 ml of B. The mixture was

shaken for 15 sec. After separation of the two layers a suitable amount (0.1 to 2.5 ml) of the upper layer was removed and diluted with E to 5 ml. 0.5 ml of D was added and mixed immediately. The colour intensity was measured at 730 nm against a reagents/water blank.

Standards of potassium dihydrogen orthophosphate in the appropriate concentration range were taken through the method.

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